AD_____

Award Number: DAMD17-99-C-9090

TITLE: Comparison of Novel and Known Neuroprotectants for
Treating Exposure to Different Types of Toxins

PRINCIPAL INVESTIGATOR: Ben A. Bahr, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut
Storrs, Connecticut 06269-4133

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

20030923 035

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2002	3. REPORT TYPE AND DATES COVERED Annual (16 Aug 01 – 15 Aug 02)

4. TITLE AND SUBTITLE
Comparison of Novel and Known Neuroprotectants for Treating Exposure to Different Types of Toxins

5. FUNDING NUMBERS
DAMD17-99-C-9090

6. AUTHOR(S):
Ben A. Bahr, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Connecticut
Storrs, Connecticut 06269-4133

E-Mail: Bahr@uconn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
Studies using cultured brain slices have found that compensatory signals are activated in response to different types of excitotoxicity/seizures related to environmental toxin or military threat agent exposure. Surprisingly, glutamate receptors, cannabinoid receptors, and proteoglycan-binding adhesion receptors activated common pathways involving MAP kinase, focal adhesion kinase (FAK), and transcription factors that facilitate endogenous repair mechanisms. Inhibition of the receptors potentiated excitotoxic vulnerability and, correspondingly, promoting receptor-mediated responses enhanced cellular and synaptic repair. The receptors seem to act through transcription regulators to explain common signaling elements including MAPK and FAK. NF-κB is one such regulator, activated in response to both excitotoxicity and receptor activation. Interestingly, NF-κB activation is biphasic; properties of the initial phase are consistent with neuroprotection and the second phase with neurodegeneration. Microarray analyses have confirmed that genes activated initially are those that promote survival, while genes activated during the delayed phase can enhance neuronal vulnerability. The latter phase may explain why months after an insult, select brain regions remain acutely vulnerable to stroke events and age-related neurodegeneration. Studies with receptor modulators are helping to identify key signal transduction pathways that lead to neuroprotection vs. those that enhance neuronal vulnerability, and what determines the direction of the signaling path.

14. SUBJECT TERMS
excitotoxicity, neurotoxins, neuronal vulnerability, neuroprotection

15. NUMBER OF PAGES
144

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

3. TABLE OF CONTENTS

4. INTRODUCTION

Our previous work has found that excessive glutamatergic activity plays a major role in excitotoxic damage related to environmental toxin or military threat agent exposure. As expected, negative modulation of glutamate receptor activity through antagonists promotes protection against excitotoxic insults (see Buchan et al., 1991; Nellgard and Wieloch, 1992; Sheardown et al., 1993). Interestingly, low-level stimulation of AMPA-type glutamate receptors has been shown to enhance neuronal survival and promote synaptic maintenance (Bambrick et al., 1995; McKinney et al., 1999). This is likely related to the fact that besides having ionotropic properties, AMPA receptors are linked to the neuroprotective mitogen-activated protein kinase (MAPK) pathway (Wang and Durkin, 1995; Hayashi et al., 1999; Bahr et al., 2002; Limatola et al., 2002). This signaling pathway can be enhanced by the positive modulators call Ampakines (Bahr et al., 2002), thereby implicating the pathway in Ampakine induction of neurotrophic factor expression (Lauterborn et al., 2000) and neuroprotection against stroke-type excitotoxicity (Bahr et al., 2002). Recent findings indicate that multiple receptor systems are linked to such endogenous repair mechanisms that are responsible for compensatory responses to injury.

AMPA receptors are known to participate in higher cognitive functions in the mammalian brain. Positive modulation of AMPA receptors by the Ampakine class of compounds selectively improves channel function by making the receptors more responsive to endogenous ligand and specific agonists (Tang et al., 1991; Arai et al., 1996; Suppiramaniam et al., 2001). The resultant increase in glutamatergic transmission has been shown to be associated with enhanced synaptic plasticity and memory retention (Staubli et al., 1992, 1994; Granger et al., 1996; Hampson et al., 1998; Lebrun et al., 2000). Thus, AMPA receptors are part of a cellular mechanism(s) that are necessary for both information processing and repair systems. Surprisingly, as in the case of AMPA class glutamate receptors, we found that cannabinoid receptors and proteoglycan-binding adhesion receptors are linked to endogenous repair mechanisms. These same two classes of receptors also have been implicated in plasticity-related memory encoding (Bahr et al., 1997; Carlson et al., 2002; Gerdeman et al., 2002), again corresponding with AMPA receptors. The multiple receptor systems, i.e. AMPA receptors, cannabinoid receptors, and adhesion receptors, were found to activate common pathways involving MAP kinase, focal adhesion kinase (FAK), and transcription factors that facilitate endogenous repair mechanisms.

5. BODY

This project has identified repair mechanisms that are able to counteract the effects of neurotoxin exposure. We have shown that AMPA-type glutamate receptors are linked to appropriate signaling events in order to prevent neuronal injury as well as enhance recovery. As reported in Bahr et al. (2002), AMPA stimulation in hippocampal slice cultures caused the selective activation of MAPK through the upstream activator MAPK kinase (MEK). Excessive glutamatergic activity through AMPA receptors is no doubt a critical feature of excitotoxic damage (Buchan et al., 1991; Sheardown et al., 1993). On the other hand, enhancing basal stimulation of AMPA receptors and associated MAPK signaling with the positive modulator Ampakine, was found to promote neuronal survival after toxic exposure (Bahr et al., 2002; Munirathinam et al., 2002). As with AMPA receptors, we recently showed that stimulation of signals through cannabinoid receptors and proteoglycan-binding adhesion receptors also mediate neuroprotection in the slice model.
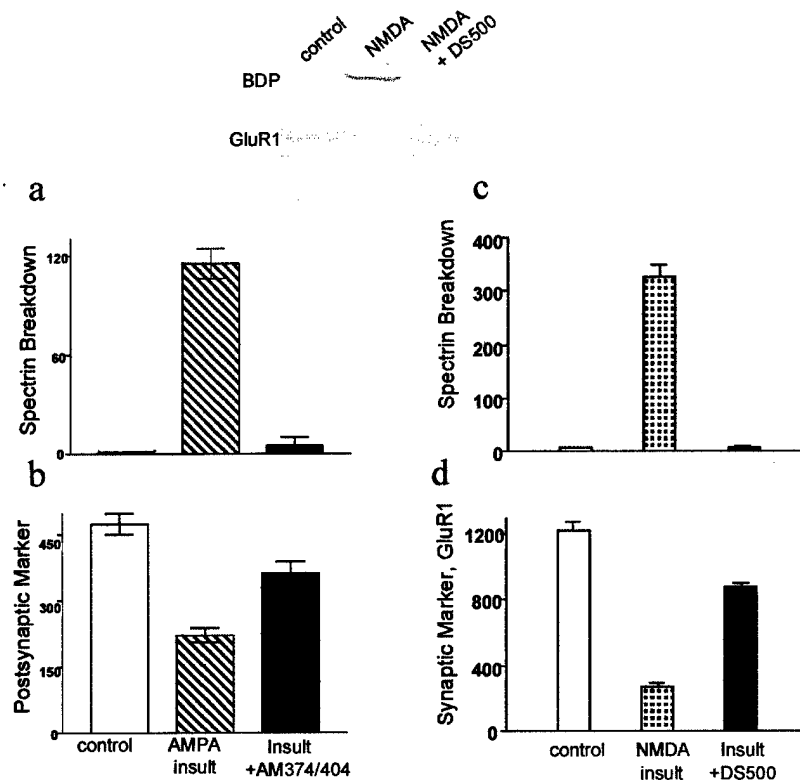
Figure 1. Cannabinoid receptors and proteoglycan-binding adhesion receptors elicit neuroprotection in hippocampal slice cultures. (a, b) Slice cultures were treated with excitotoxic level of AMPA for 30 min, and the 24-h recovery period was in the absence or presence of 0.1 μM AM374 and 10 μM AM404, a drug combination that enhances endogenous cannabinoid signaling. (c, d) Slices were treated with excitotoxic level of NMDA for 20 min, and the 24-h recovery period was in the absence or presence of 10 nM DS500 in order to stimulate proteoglycan-binding adhesion receptors. Slice samples were assessed by immunoblot for spectrin BDP (a, c) and the postsynaptic marker GluR1 (b, d); data represent mean integrated densities ± SEM. Recovery levels were significant at p<0.001.

Enhancing signaling through cannabinoid receptors and proteoglycan-binding adhesion receptors was tested for neuroprotection in the slice model. The excitotoxic action of AMPA and NMDA caused lasting spectrin breakdown (BDP) mediated by the excitotoxic protease calpain (Fig. 1a and 1c; see immunoblot at top), as well as synaptic decline (Fig. 1b and 1d). Enhancing endogenous cannabinoid signaling can be achieved by blocking the degradation and recycling transport of endocannabinoid ligands with the inhibitors AM374 and AM404 (Beltramo et al., 1997; Deutsch et al., 1997; Gifford et al., 1999; Giuffrida et al., 2001; Gerdeman et al., 2002). Increasing endocannabinoid signaling in this way almost completely eliminated the spectrin breakdown and the reductions in pre- and postsynaptic markers produced by the excitotoxic exposure (Fig. 1a and 1b). In Figures 1c and 1d, similar neuroprotection was found when proteoglycan-binding adhesion receptor signaling was stimulated with the glycosaminoglycan dextran sulfate of 500 kDa (DS500). These results are of particular significance because the receptor enhancing conditions of AM374/404 and DS500 were not subjected to slice cultures until after the excitotoxin exposure period. The level of recovery produced by post-insult modulation of cannabinoid receptors and proteoglycan-binding adhesion receptors was comparable to the protection elicited by pre-incubation with Ampakine.
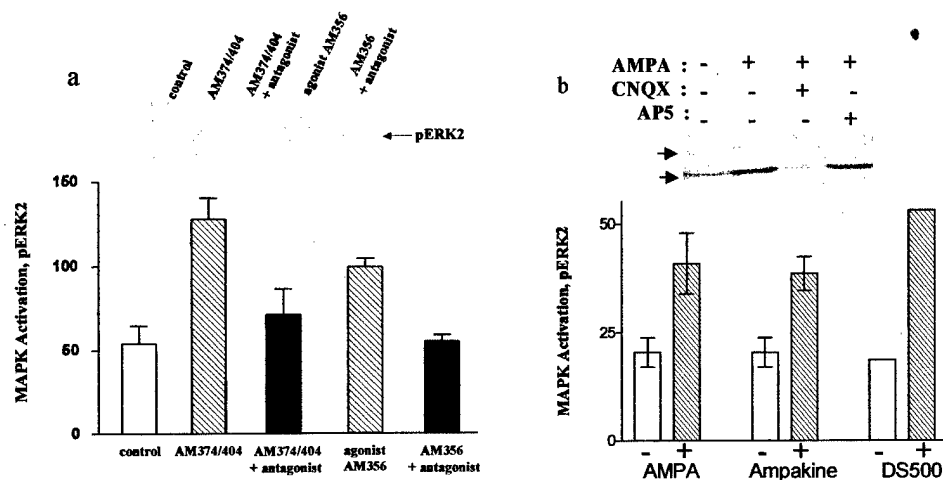
5

Figure. 2. Multiple receptor systems are linked to the MAPK signaling pathway. The different types of receptors were stimulated, and slices were assessed by immunoblot for the phosphorylated active ERK isoforms of MAPK (shown are mean integrated density values of pERK2 ± SEM). (a) Cannabinoid receptors were stimulated for 60 min with the agonist AM356 (100 μM) or with the AM374/404 combination used in Figure 1 that enhances endocannabinoid signaling. The resultant 1-2-fold increase in MAPK activation was blocked by the cannabinoid receptor antagonist AM281 (p<0.01). (b) Slices were treated with or without AMPA for 3 min, Ampakine modulator of the AMPA receptors for 60 min, and 10 nM DS500 to stimulate proteoglycan-binding adhesion receptors for 60 min. In the blot, untreated slices and slices exposed to AMPA for 2 min in the absence or presence of CNQX or AP5 were assessed for pERK isoforms.

The same receptor systems shown to elicit neuroprotection in the slice model also activated common signaling pathways. As recently reported for AMPA receptor stimulation (Bahr et al., 2002), MAPK isoforms (ERK1/2) are selectively activated when phosphorylated on Thr and Tyr residues of their catalytic core by the kinase MEK (see blot above Fig. 2b). Stimulation by AMPA caused rapid MAPK activation as indicated by enhanced staining of ERK1 and ERK2 with an antibody that recognizes the dually phosphorylated isoforms. The effect was block by AMPA receptor antagonist CNQX but not by other glutamate receptor antagonists. Similar MAPK activation occurred when cannabinoid signaling was stimulated with the agonist AM356 as well as with the AM374/404 combination that enhances endocannabinoid signals (Fig. 2a). The resultant increase in MAPK activation was blocked by the cannabinoid receptor antagonist AM281. Interestingly, MAPK also was activated to a similar degree when AMPA receptors were subjected to allosteric modulation alone with Ampakine CX516, or when proteoglycan-binding adhesion receptors were stimulated with the same level of DS500 that produced neuroprotection (Fig. 2b).
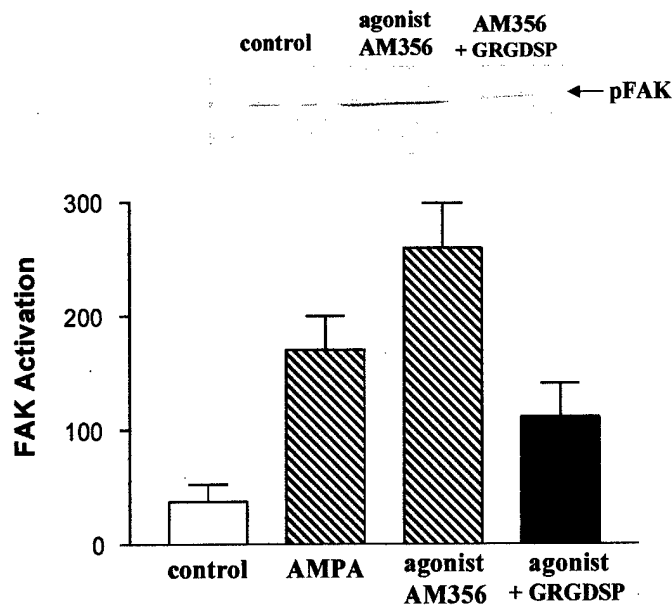
6

Figure. 3. AMPA receptors and cannabinoid receptors activate FAK. The different types of receptors were stimulated with agonist for 20-60 min, and slices were assessed by immunoblot for the phosphorylated active form of FAK (mean integrated density ± SEM). The resultant 3-5-fold increase in FAK activation was blocked by the integrin antagonist GRGDSP.

In addition to the MEK/MAPK activation events, multiple receptor systems also have a common influence on other components of protection-mediating signaling pathways. We recently found that different receptors activate not only the MAPK pathway but also the upstream element focal adhesion kinase (FAK), the primary mediator of integrin signals that regulate cell viability. Through their interaction with the extracellular matrix, integrins can translate changing conditions in the extracellular environment into FAK signals capable of modulating intracellular signal transduction. As shown in Figure 3, the activated form of FAK was labeled by antibodies to phosphoFAK (pFAK), and the staining was increased by stimulating AMPA receptors with AMPA or cannabinoid receptors with AM356 agonist. The FAK activation as well as MAPK activation events were blocked by the GRGDSP peptide that selectively inhibits integrin-mediated adhesion responses.

Disruption of endogenous repair signals can have dramatic effects on the relative vulnerability of brain tissue. Blocking the different components of the AMPA receptor-MAPK pathway was previously found to enhance vulnerability to conditions that were normally well tolerated (Bahr et al., 2002). Enhanced neuronal vulnerability could explain why long after exposure to an environmental toxin or military threat agent, select brain regions can remain acutely vulnerable to stroke events or age-related neurodegenerative processes. Chronic exposure of toxins at low levels could very well be disrupting endogenous repair systems although alone the exposure does not elicit overt pathogenic changes. Thus, it is important to study how neuronal vulnerability can be enhanced. Long-term brain slice cultures are ideal for such a task since they exhibit similar pathogenic responsiveness as found *in vivo* but in the absence of systemic variables (Bergold et al., 1993; Bahr, 1995; Bahr et al., 2002; Bendiske et al., 2002). More importantly, the stability of the cultured slice renders it a good model for studying pathogenic processes that may be drawn out and expressed only by a change in vulnerability to additional stress factors (see Bahr et al., 1994, 1998; Bendiske et al., 2002). Since our previous work showed that vulnerability is enhanced by specific inhibitors of MEK and MAPK, additional studies were conducted in slices treated to disrupt integrin-mediated adhesion responses including FAK activation.
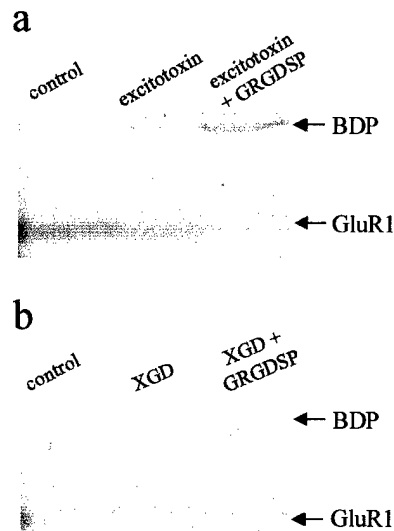
7

Figure 4. Blocking integrin-type adhesion responses increases vulnerability to different types of insults. Cultured slices were treated with (a) excitotoxic levels of AMPA for 20 min or (b) a sub-pathogenic GD level for 24 h in the absence or presence of the integrin antagonist GRGDSP. Slice samples were analyzed 24 h post-insult for calpain-mediated spectrin breakdown product (BDP) and the synaptic marker GluR1.

FAK activation was disrupted by the GRGDSP integrin antagonist as shown in Figure 3. Brain slices pretreated with GRGDSP were tested for their pathogenic responses to a brief exposure to an excitotoxin (Fig. 4a) or to a 24-h exposure to a sub-pathogenic concentration of soman (GD) (Fig. 4b). In both cases, the insults produced more evidence of cytoskeletal breakdown and synaptic decline than is normally expressed in the absence of the integrin antagonist. It appears, then, that endogenous maintenance pathways involve adhesion responses linked to FAK-MEK-MAPK signaling. Also, low-level GD exposure during a time when brain circuits and their signaling are compromised can indeed lead to excitotoxic processes; such processes are exemplified by calpain-mediated spectrin breakdown and synaptic decline, known characteristics of varying types of excitotoxicity (Vanderklish and Bahr, 2000; Bahr et al., 2002).

Further worked looked at whether brain tissue can experience increased vulnerability following a brief period of neuronal stress which can come from excitotoxic conditions including hypoxia, ischemia, mild stroke, or head trauma. We used trimethyltin (TMT), an environmental toxin capable of inducing excitotoxic processes after 4-5 h in the slice model (Bahr et al., 2002; Munirathinam et al., 2002). The short 1-h TMT exposure used in Figure 5 (middle pair of samples) produced little evidence of spectrin breakdown (BDP) or reductions in presynaptic marker synaptophysin (Syn) or postsynaptic marker GluR1 assessed 24 h later. However, a robust BDP response (5-fold increase) and synaptic decline (-30-50%) were evident when such TMT exposure was followed by a single sub-pathogenic application of GD (Fig. 5, rightmost pair). Slices were assessed for pathogenic changes 24 h after the single GD infusion at 0.1 µM. This low level of GD alone did not induce degenerative events after the 24-h period (see leftmost pair). Thus, brain tissue compromised by mild insults is made more vulnerable to GD exposure. Further experiments went on to show the reverse is true. Low-level GD exposure for a week potentiated neuronal vulnerability to acute toxic insults. Interestingly, positive modulation of glutamatergic signaling linked to MAPK during the week-long GD exposure was neuroprotective, completely abolishing pathogenic changes induced by the subsequent insult (2002 abstract). These data indicate that prolonged exposure to dilute GD enhances neuronal vulnerability, and endogenous repair pathways act against this.
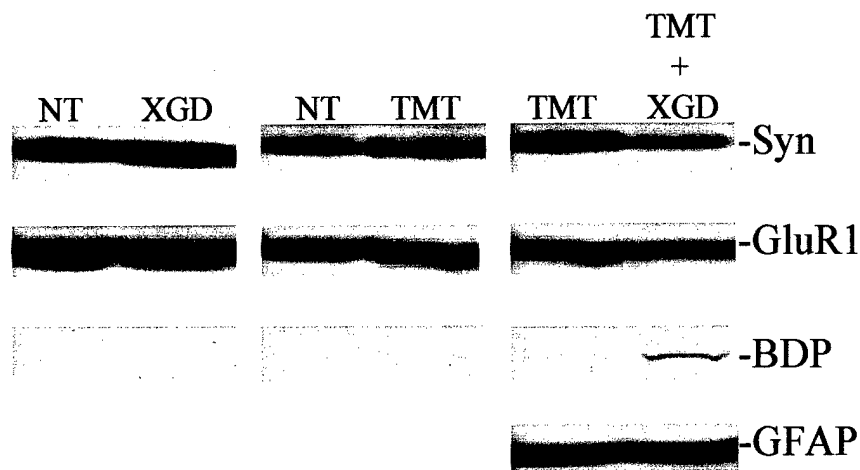
Figure 5. Cultured hippocampal slices exhibit increased vulnerability following a brief excitotoxic insult. Slices were exposed to 100 μM TMT for 1 h (middle pair), and 24 h later immunoblots of slice samples showed little evidence of spectrin breakdown (BDP) or reductions in synaptophysin (Syn) or GluR1 as compared to non-treated control slices (NT). Cytoskeletal and synaptic degeneration were evident when the TMT exposure was followed by a 24-h application of 0.1 μM GD (see rightmost pair). This low level of GD alone did not induce degenerative events after the 24-h period (leftmost pair).

Studies were done in an attempt to find possible drug combinations that could improve neuroprotection against the increased vulnerability. The anticonvulsant drug huperzine A and the anti-inflammatory drug NS398 produced marginal protection as compared to that produced by enhancing endogenous repair signals. Broad-spectrum anti-inflammatories were previously shown to elicit only minor beneficial effects. Following mitochondrial toxicity as with excitotoxin exposure, some evidence of additive neuroprotection was found between the AMPA receptor-MAPK pathway modulator Ampakine and NS398, a cyclooxygenase-2 inhibitor (Table 1). However, across different synaptic markers, additive recovery was not consistent. This leads to the conclusion that endogenous repair signals through receptors like AMPA receptors, cannabinoid receptors, and adhesion-type receptors are sufficient to protect against toxin exposures, and that pursuing combinations with alternate protection avenues is not warranted.

Agent	Extent of:	GluR1 Recovery	Synapsin Recovery	Synaptophysin Recovery
none		−	−	−
Ampakine		+++	+++	+++
Ampakine+NS398		++++	+++	++
NS398		+++	+++	++

Table I. Positive modulation of the AMPA receptor-MAPK pathway with Ampakine was tested with or without the anti-inflammatory agent NS398 for inducing recovery of different synaptic markers. The three synaptic markers were reduced 60-90% following the exposure to mitochondrial toxin (3NP) for 24 h. Recovery was evaluated by comparing marker levels in slices that did not receive any protective agent.

9

The advantage of using the cultured brain slice model to study cellular repair systems is apparent from the model being unlike dissociated neurons and very similar to adult brain tissue, expressing the characteristic circuitry, integrity, and organization of neuronal layers and dendritic fields (Bahr et al., 1995a). In addition, the same compensatory signaling whose modulation enhances recovery in the slice model is also neuroprotective and can be similarly exploited *in vivo* (Bahr et al., 2002). Neuroprotection is efficiently assessed in slice cultures since the model allows for controlled toxic conditions and the ability to conduct signaling effects in the absence of systemic variable. Neurotoxicity measures of particular usefulness are the loss of synaptic proteins and the activation of the calcium-dependent protease calpain. Calpain-mediated cytoskeletal damage and reduced expression of synaptic markers represent early neurodegeneration, thus they are valuable for identifying potential compensatory pathways (Harry et al., 1985; Brock and O'Callaghan, 1987; Bahr et al., 1994, 1998, 2002; Vanderklish and Bahr, 2000; Bendiske et al., 2002; Bendiske and Bahr, 2002).

With the slice model, cell-surface signal transduction through the link between different receptors and MAPK has been implicated as an important step in neuronal maintenance. The multiple receptor types seem to act through transcription regulators to explain common signaling elements including MAPK and FAK. Inducible transcription factors are important in communicating changes in the cellular environment into specific signaling responses. Nuclear factor-κB (NF-κB) is one such factor, activated in response to both excitotoxicity and receptor activation. This transcription factor underlies the cell's ability to respond to a wide range of stimuli ranging from growth and differentiation to toxic insult. Correspondingly, studies with the slice model have suggested that NF-κB activation 1) represents a compensatory repair response activated early after the onset of excitotoxic conditions (Caba et al., 2002) and 2) also occurs as part of the pathogenesis elicited when AMPA receptor-MAPK signaling is disrupted for an extended period (Bahr et al., 2002). The transcription factor NF-κB is known to be activated in response to certain types of pathogenic episodes (Mattson et al., 2000; Bahr et al., 2002).

As illustrated in our studies, there is an inherent complexity with regards to NF-κB activation, its involvement being implicated in both promoting cell damage as well as cell survival. Components of such a dual role have been seen mediated by different stimuli in non-brain tissue, however, NF-κB activation in the brain in fact leads to protective and pathogenic pathways as a result of a single type of stimuli. Thus, while there are studies implicating NF-κB in protective compensatory signaling in response to excitotoxicity (Grilli et al., 1996; Clemens et al., 1997; Qin et al., 1998; Nakai et al., 2000; Bachis et al., 2001; Caba et al., 2002), there also exists studies that point to the pathogenic role played by NF-κB (Goodman and Mattson, 1996; Mattson, 1997; Tamatani et al., 2000; Lipsky et al., 2001; Rosenberger et al., 2001). Together with our current work, these studies lead to the idea that NF-κB activation initially triggers compensatory signaling pathways, but later is capable of enhancing neuronal vulnerability perhaps to apoptotic mechanisms.

In correspondence to this idea, we found that NF-κB activation is biphasic in response to an excitotoxic insult. We evaluated the activation profile of NF-κB in organotypic hippocampal slice cultures subjected to a 20-min NMDA exposure. The hippocampus is a part of the brain that is particularly targeted by neuropathologic events such as stroke-type and toxin-induced excitotoxicity. The brief insult resulted in a biphasic activation profile for NF-κB as determined by EMSA, consisting of an initial 4-fold increase, followed by a second phase of activation evident 24 h post-insult (ANOVA $p < 0.0001$). See Figure 6 for a diagram of the biphasic response.
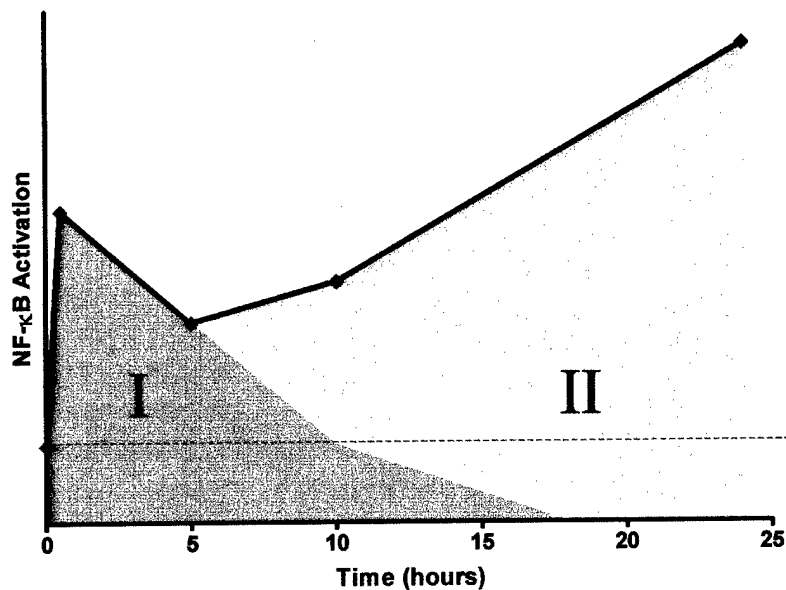
Figure 6. NF-κB is activated in a biphasic manner following a brief 20 min NMDA exposure.

Such biphasic activation could explain the dual role of NF-κB observed by numerous studies resulting in the controversial implication of NF-κB in both protection and pathogenesis. The results support the hypothesis that NF-κB activation is comprised of two distinct phases in response to excitotoxicity in the hippocampus, and that the phases promote opposing signaling pathways.

Further characterization of NF-κB activation required the assessment of cellular response pathways that potentially have different effects on the two phases. Interestingly, properties of the initial phase are consistent with neuroprotection and the second phase with degeneration. For instance, recent data have shown that MEK activation resulting in increased phosphoERK isoforms corresponds only with phase I of NF-κB activation (Fig. 7b). Accumulating evidence suggests that the MEK-MAPK pathway is involved in promoting compensatory mechanisms during excitotoxicity (Bonni et al., 1999; Bahr et al., 2002; Mitin et al., 2001; Rakhit et al., 2001), and also has been implicated in NF-κB activation (Briant et al., 1998; Chen and Lin, 2001). Since the increase in the MEK-MAPK response was observed to coincide only with phase I of NF-κB activation, this suggests that NF-κB activation in phase II may be mediated by a MEK-independent pathway.

Further evidence that MEK activation is one of the distinguishing characteristics of the two phases is shown in Figure 7a. This was from work that found that drug treatments can be used to influence the phases differentially, thereby further establishing that the two phases are distinct. Incubating the slice cultures with the neuroprotectant Ampakine following the brief NMDA insult results in the enhancement of NF-κB activation selectively in phase II. Ampakine modulates the AMPA receptor-MAPK pathway. Interestingly, neuroprotectant-induced signaling corresponds with the second phase and actually causes an additive level of transcription factor activation. Thus, the original idea that neuroprotection is mediated through blockage of NF-κB activation is not true. The first phase of activation after a toxic insult appears to represent a compensatory repair response,

11

while the second phase of the NF-κB response is consistent with delayed pathogenesis that may explain the increased vulnerability following chronic exposure to mild toxin levels.
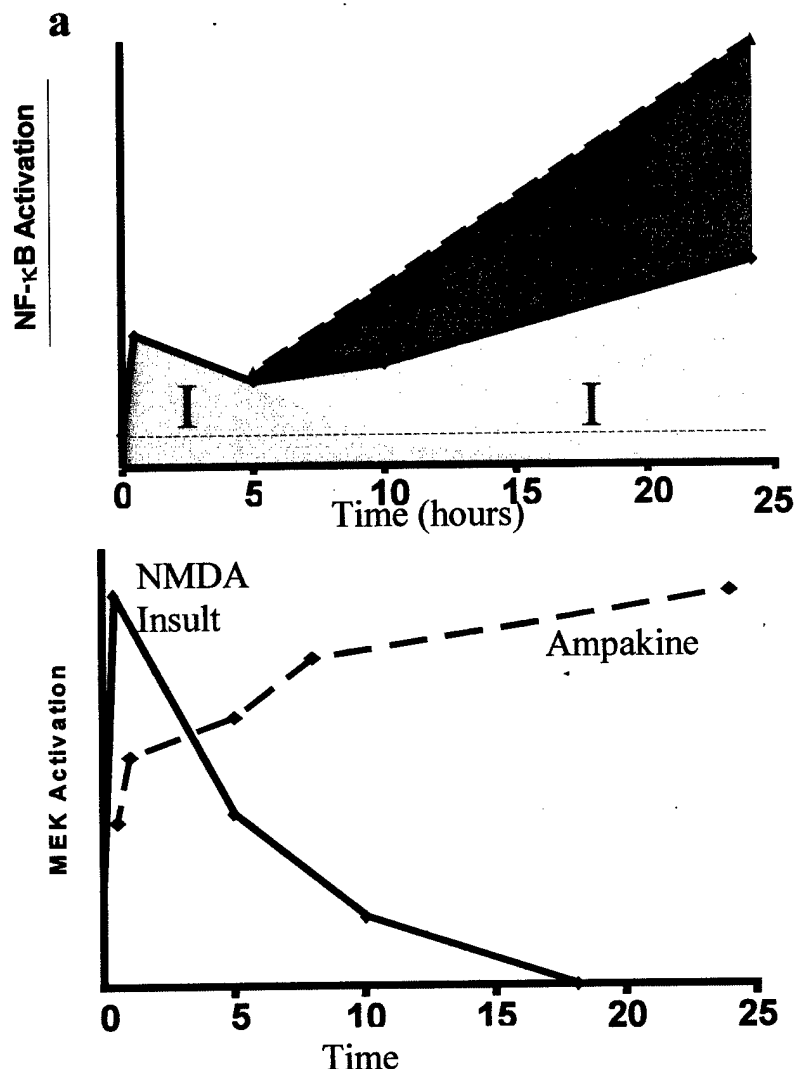
Figure 7. Neuroprotective drug (Ampakine) that modulates the AMPA receptor-MAPK pathway, enhances NF-κB activation in phase II (a) and not in phase I. When used alone Ampakine activates MEK over an extended period (b) unlike transient activation observed following a brief NMDA insult.

As shown in Figure 7b, the Ampakine neuroprotectant alone causes prolonged activation of MEK. The neuroprotectant has an additive effect on NF-κB activation in phase II but not in phase I, likely due to the fact that MEK is already fully activated in response to the NMDA insult in the first phase. Thus, these data suggest that NF-κB activation due to brief excitotoxicity consists of a MEK-dependent phase I and a MEK-independent phase II. Such studies with receptor modulators are helping to identify key signal transduction pathways that lead to neuroprotection vs. those that enhance neuronal vulnerability, and what determines the direction of the signaling path. To further

12

establish this distinction, other treatments that activate MEK systems or, alternatively that diminish such systems, have been assessed. Consequently, this work begins to establish that there are two distinct phases underlying NF-κB's activation profile.

With our latest work, we are beginning to understand how a transcription factor can have a dual role in responding to a single stimulus. Further studies are required to better understand the separate pathways and to address whether the two phases lead to differential gene transcription. There are checkpoints and redundancies that enable the correct signal to be carried out and hence, activation of a single transcription factor can be achieved via multiple receptors, or cascades, to result in gene transcription. Resolving the two phases as distinct will require full characterization and will help explain the dual role of NF-κB. Unraveling the nature of how NF-κB activation leads to opposing responses will shed light into the orchestration of compensatory responses as well as the cellular compromise that results in neuronal vulnerability.

We recently incorporated gene expression analysis to determine whether NF-κB regulated genes exhibit differential expression in phase I versus phase II. Because of the dual role of NF-κB in survival and pathogenic pathways, representative genes of each pathway were monitored to help establish that phase I and phase II facilitate opposing signaling mechanisms. NF-κB regulates a variety of genes including those involved in survival, growth, differentiation, and cell maintenance, as well as inflammation and cell death mechanisms (Pahl, 1999; Barkett and Gilmore, 1999; Sonenshein, 1997; Perkins, 2000). Bcl-2 is an anti-apoptotic member of the bcl-2 family that is widely expressed in the nervous system (Boise et al., 1993; Gonzalez-Garcia et al., 1995). It's expression was found to be increased early after the excitotoxic insult by RT-PCR, thus a protective gene is activated in phase I. Bax, also a member of the bcl-2 family, is a pro-apoptotic protein (Oltvai et al., 1993; Ananth et al., 2001). Correspondingly, the expression of this pathogenic gene was increased only in phase II.

Using microarrays, we monitored thousands of genes, focusing on genes that promote survival vs. those that can enhance neuronal vulnerability. Neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are involved in neuroprotection (Glazner and Mattson, 2000; Lipsky et al., 2001), whereas pro-inflammatory proteins have been implicated in progression of pathology (Adams et al., 1996; Koistinaho et al., 1999; Hewett et al., 2000). Microarray analyses have confirmed that genes activated initially after the excitotoxic exposure are those that promote survival, while genes activated during the delayed phase are consistent with enhancing neuronal vulnerability (Fig. 8). A correlation also exists for which genes are reduced in expression in the different phases. The latter phase of NF-κB activation may explain why months after an insult, select brain regions remain acutely vulnerable to mild pathogenic episodes and accelerated neurodegeneration. In conclusion thus far, NF-κB activation by an excitotoxic insult is biphasic, and properties of the initial phase are consistent with neuroprotection and the second phase with degeneration.

Low-level exposure to agents such as TMT or GD could make tissue more vulnerable by disrupting signaling elements of the first phase of compensatory responses involving transcription factors. The dual role of NF-κB following exposure to toxic agents can be resolved by examining related signaling pathways as well as determining the genes involved. This will expand our fundamental understanding of the part played by the transcription factor.
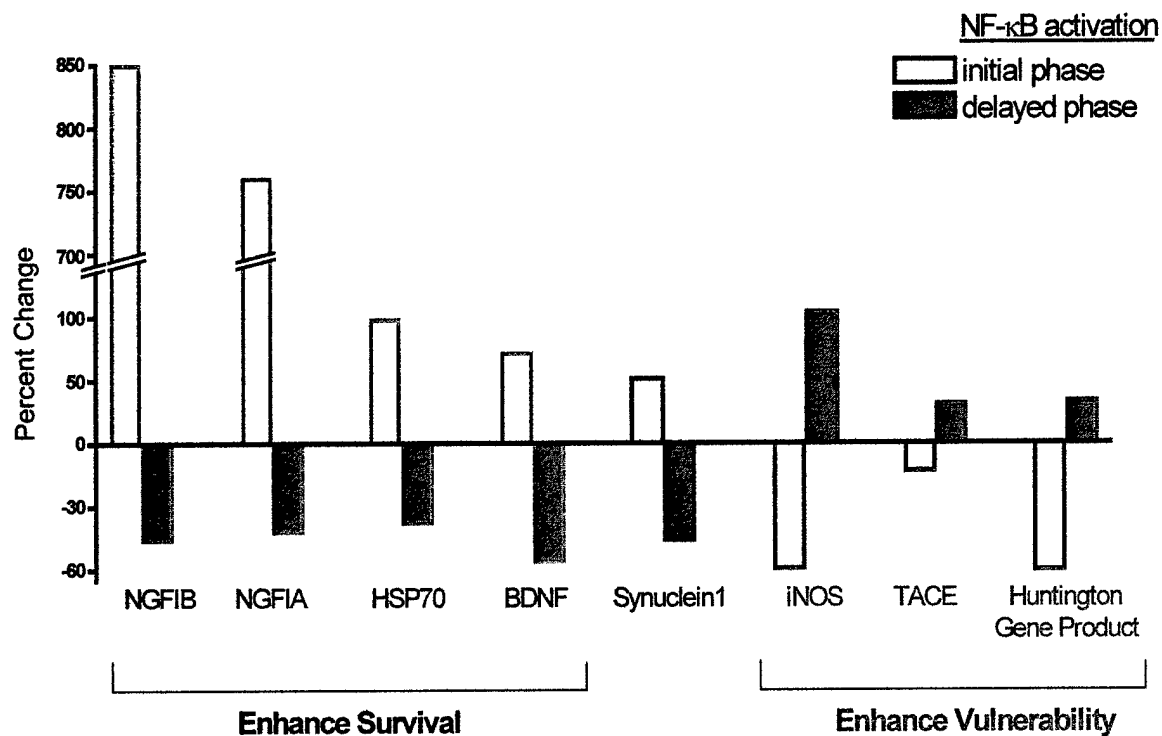
Figure 8. Microarray analyses found evidence that genes activated during NF-κB's first phase are consistent with pro-survival genes (green), while genes activated during the delayed phase of activation are those that enhance neuronal vulnerability (red). A correlation also exists for which genes are reduced in expression in the different phases.

6. KEY RESEARCH ACCOMPLISHMENTS

❑ To be thorough and to extend Specific Aim I for completion, we have identified two additional classes of receptors that elicit compensatory repair signals similar to those of the AMPA receptor-MAPK pathway. With the involvement of multiple receptor systems, we have a better ability to identify common signaling elements that potentially point to endogenous repair mechanisms.

❑ With the conclusion of Specific Aim I, we established that enhancing signaling through AMPA receptors, cannabinoid receptors, or adhesion-type receptors leads to neuroprotection via compensatory signaling in a model of neurotoxin exposure. Although there is occasional evidence of additive effects between distinct avenues of neuroprotection, e.g., enhancing MAPK compensatory responses vs. anti-inflammatory action, much of the findings indicate that efficient repair is elicited by compensatory signals alone. In addition, up-regulation of endogenous repair signaling facilitated long-term cell survival and synaptic maintenance. As reported in our 2002 publication (*Exp. Neurol.*), modulation of glutamatergic signaling linked to MAPK leads to reduction in neuronal damage measured 7-10 days after an excitotoxic insult *in vitro* and *in vivo*.

❑ From experiments of Specific Aim II, we further established that endogenous repair signals activated through different receptor systems protect against the effects of environmental toxin or military threat agent exposure. One study has been accepted for publication, and another is in preparation. To be thorough, we also added a mitochondrial toxin (3NP) to our list of toxic agents. Mitochondrial dysfunction has been implicated in stroke and age-related diseases such as Parkinsons, and may be involved in enhancing neuronal vulnerability after low-level toxin exposure. Recent work shows the same neuroprotectants that act against TMT and GD also protect against the action of the mitochondrial toxin.

❑ With the conclusion of Specific Aim II, we established that neuroprotectant can be applied to brain tissue after the toxic exposure and still elicit pronounced levels of cellular repair and synaptic recovery. More importantly, we discovered that neurons become acutely vulnerable to damage after low-level chronic exposure to toxic agents such as TMT and GD. While few pathogenic markers were evident in brain tissue following the chronic exposure, the resultant increased vulnerability allowed a subsequent minor insult to produce marked levels of cellular damage and synaptic decline.

❑ As part of Specific Aim III, we found that endogenous repair responses involving MAPK signaling are still effective neuroprotection pathways when stimulated by receptor agonists 3 or even 6 hours after an excitotoxic insult. Thus, there appears to be a prolonged window of opportunity to activate recovery systems after an insult in order to elicit brain repair.

❑ Furthering the third Specific Aim, we recently found that glutamate receptors, cannabinoid receptors, and proteoglycan-binding adhesion receptors all activate not only the MAPK pathway but also the upstream enzyme focal adhesion kinase (FAK), the primary mediator of integrin signaling. In addition, blocking integrin signaling with antagonist disrupted MAPK and FAK activation and, correspondingly, enhanced neuronal vulnerability. These results indicate that integrin-type adhesion responses contribute to endogenous signaling pathways underlying cellular recovery.

15

□ Specific Aim III experiments found that the multiple receptor systems being studied also activate a transcription factor known to facilitate endogenous repair mechanisms. NF-κB is the transcription factor activated in response to both excitotoxicity and to receptor activation. NF-κB activation by an excitotoxic insult is biphasic, however, and properties of the initial phase are consistent with neuroprotection and the second phase with degeneration. Interestingly, neuroprotectant-induced signaling corresponds with the second phase and actually causes an additive level of transcription factor activation. Thus, the original idea that neuroprotection is mediated through blockage of NF-κB activation is not true. The first phase of activation after a toxic insult appears to represent a compensatory repair response, while the second phase of the NF-κB response is consistent with delayed pathogenesis that may explain the increased vulnerability following chronic exposure to mild toxin levels.

□ Recent work under Specific Aim III utilized microarray analyses to find evidence that genes activated during NF-κB's first phase are consistent with pro-survival genes, while genes activated during the delayed phase of activation are those that enhance neuronal vulnerability. Studies are continuing with receptor modulators to help identify signaling pathways that lead to neuroprotection vs. those that enhance neuronal vulnerability.

7. REPORTABLE OUTCOMES

□ MANUSCRIPTS (attached at end of appendices)

1. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, and Rogers G (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp Neurol* **174**:37-47.

2. Caba E, Brown QB, Kawasaki B, and Bahr BA (2002) Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. *J Neuroscience Res* **67**:787-794.

3. Bendiske J, Caba E, Brown QB, and Bahr BA (2002) Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J Neuropathol Exp Neurol* **61**:640-650.

4. Vicente JS, Munirathinam S, and Bahr BA (2002) Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age. *J Mol Biol Biotech* **3**:1-9.

5. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of AMPA-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol*, in press.

6. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem*, in press.

□ ABSTRACTS (included in appendices)

1. Caba E and Bahr BA, Biphasic response of NF-κB to stroke-type pathogenesis in organotypic hippocampal slice cultures, *GRASP Pharmacy Research Conference* (2001).

2. Bendiske J and Bahr BA, Characterization and slowing of the protein deposition and synaptic decay cascade in hippocampal slice cultures, *Second Neurobiology Conference: Identifying Accelerators and Brakes of Brain Aging* (2001).

3. Caba E, Giardina C, Rogers G, Bahr BA, Ampakine effects indicate that the biphasic NF-κB response to excitotoxicity denotes two opposing signaling pathways, *Soc. Neurosci. Abstr.*, **27**, 867.2 (2001).

4. Suppiramaniam V, Subramaniam T, Kanju PM, Kleene R, Moss A, Premkumar L, Brown Q, Bahr BA, and Dityatev A, Single channel recordings from mossy fiber synaptosomes: Evidence for the presence of high-affinity synaptic AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 502.10 (2001).

5. Subramaniam T, Kleene R, Manivannan K, Dityatev A, Bahr BA, and Suppiramaniam V, Polysialic acid modulates single channel properties of synaptosomal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 610.5 (2001).

6. Chicoine L, Suppiramaniam V, Gianutsos G, Kawasaki BT, and Bahr BA, Sulfated polysaccharides modulate calcium permeability and binding properties of hippocampal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 480.6 (2001).

7. Bahr BA, Bendiske J, Michaelis ML, Georg G, Caba E, and Brown Q, Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders, *Soc. Neurosci. Abstr.*, **27**, 581.2 (2001).

8. Bendiske J and Bahr BA, Positive lysosomal modulation reverses the PHF-tau accumulation ◇ microtubule destabilization ◇ transport failure ◇ synaptic deterioration cascade, *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001).

9. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, The majority of cell death occurs early after intracerebral grafting of ventral mesencephalic tissue: A role for calpain activation. *Soc. Neurosci. Abstr.*, **27**, 197.4 (2001).

10. Bahr BA, Caba E, Giardina C, Brown QB, and Bendiske J, Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways, *Mol. Biol. Cell*, **12** (Suppl.), 2233 (2001).

11. Munirathinam S and Bahr BA, Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *The Toxicologist* (Society of Toxicology), **58**, 1551 (2002).

□ PRESENTATIONS

1. Society for Neuroscience Annual Meeting: "Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders", San Diego, California (2001).

2. American Society of Cell Biology Annual Meeting: "Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways," Washington D.C. (2001).

3. Society of Toxicology Annual Meeting: " Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability", Nashville, Tennessee (2002).

4. International Conference on Alzheimer's Disease: "Activation of the Lysosomal System Reverses Neuropathogenesis in the Slice Model of Protein Deposition", Stockholm, Sweden (2002).

8. CONCLUSIONS

Glutamate receptors, cannabinoid receptors, and proteoglycan-binding adhesion receptors can transmit extracellular signals to the nucleus through MAPK and FAK signaling, and our work shows that, in this way, the receptors produce a compensatory pathway representing a key neuroprotection avenue. Inhibition of the receptors was shown to potentiate neuronal vulnerability and, correspondingly, promoting receptor-mediated responses enhanced cellular and synaptic repair. The studies used the cultured brain slice model, which has been valuable for identifying common compensatory signals activated in response to different types of neurotoxic insults. With an increased understanding of signaling pathways that lead to neuroprotection vs. those that enhance neuronal vulnerability, we are now at the point where key signal transduction elements can be identified that determine the direction of the signaling path.

Long after exposure to an environmental toxin or military threat agent, select brain regions can remain acutely vulnerable to stroke events and age-related neurodegenerative disorders such as Parkinson's disease. Similarly, low-level chronic exposure of slice cultures to toxic agents leads to increased vulnerability to subsequent insults. Compensatory responses including the activation of select transcription factors appear to be involved in offsetting the increased vulnerability. It may indeed be the case that exposures to differently acting toxicants can be treated with the same protection strategy that exploit endogenous repair systems.

9. REFERENCES

Adams J, Collaco-Moraes Y, de Belleroche J (1996) Cyclooxygenase-2 induction in cerebral cortex: an intracellular response to synaptic excitation. J Neurochem 66:6-13.

Ananth C, Thameem Dheen S, Gopalakrishnakone P, Kaur C (2001) Domoic acid-induced neuronal damage in the rat hippocampus: Changes in apoptosis related genes (Bcl-2, Bax, caspase-3) and microglial response. J Neurosci Res 66:177-190.

Arai, A., Kessler, M., Rogers, G., and Lynch, G. (1996). Effects of a memory enhancing drug on DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents and synaptic transmission in hippocampus. J. Pharmacol. Exp. Ther. 278, 627-638.

Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G, Mocchetti I (2001) Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. J Neurosci 21:3104-3112

Bahr BA (1995) Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. J Neuroscience Res 42:294-305.

Bahr BA, Abai B, Gall C, Vanderklish PW, Hoffman KB, Lynch G (1994) Induction of β-amyloid containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 129:1-14.

Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Singh Mutneja M, Gall C, and Hoffman KB (1995a) Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5:425-439.

Bahr BA, Staubli U, Xiao P, Chun D, Zhan-Xin J, Esteban ET, and Lynch G (1997) Arg-Gly-Asp-Ser-selective adhesion and the stabilization of long-term potentiation: Pharmacological studies and the characterization of the candidate matrix receptor synaptegrin-1. J Neuroscience 17:1320-1329.

Bahr BA, Hoffman KB, Yang AJ, Hess US, Glabe CG, and Lynch G (1998) Amyloid β-protein is selectively internalized by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of APP. J Comp Neurol 397:139-147.

Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, Rogers G. (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol 174(1):37-47.

Bambrick, LL, PJ Yarowsky, and BK Krueger 1995. Glutamate as a hippocampal neuron survival factor: an inherited defect in the trisomy 16 mouse. *Proc. Natl. Acad. Sci. USA* **92**: 9692-9696.

Barkett M, Gilmore TD (1999) Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 18:6910-6924.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D. (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277(5329):1094-1097.

Bendiske, J., and Bahr, B. A. (2002). Activation of the lysosomal system: A compensatory response against protein deposition and associated synaptopathogenesis. J. Neuropathol. Exp. Neurol., in press.

Bendiske J, Caba E, Brown QB, Bahr BA. (2002) Intracellular deposition, microtubule destabilization, and transport failure: an "early" pathogenic cascade leading to synaptic decline. J Neuropathol Exp Neurol 2002 61:640-50.

Bergold PJ, Casaccia-Bonnefil P, Zeng XL, Federoff HJ. (1993) Transsynaptic neuronal loss induced in hippocampal slice cultures by a herpes simplex virus vector expressing the GluR6 subunit of the kainate receptor. Proc Natl Acad Sci U S A 90(13):6165-9.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, and Greenberg ME (1999) Cell Survival Promoted by the Ras-MAPK Signaling Pathway by Transcription-Dependent and -Independent Mechanisms. Science 286(5443), 1358-1362.

Briant L, Robert-Hebmann V, Sivan V, Brunet A, Pouyssegur J, Devaux C (1998) Involvement of extracellular signal-regulated kinase module in HIV- mediated CD4 signals controlling activation of nuclear factor-kappa B and AP-1 transcription factors. J Immunol 160:1875-85.

Brock TO, O'Callaghan JP. (1987) Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary acidic protein are associated with chemical-induced injury to the rat central nervous system. J Neurosci 7(4):931-42.

Buchan AM, H Li, S Cho, WA Pulsinelli 1991. Blockade of AMPA receptor prevents CA1 hippocampal injury following transient forebrain ischemia. *Neurosci. Lett.* **132**: 255-258.

Caba E, Brown QB, Kawasaki B, Bahr BA. (2002) Peptidyl α-keto amide inhibitor of calpain blocks excitotoxic damage without affecting signal transduction events. J Neurosci Res 67(6):787-94.

Carlson G, Wang Y, Alger BE. Endocannabinoids facilitate the induction of LTP in the hippocampus. Nat Neurosci. 2002 5:723-4.

Chen BC, Lin WW (2001) PKC- and ERK-dependent activation of I kappa B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation. Br J Pharmacol 134:1055-65.

Clemens JA, Stephenson DT, Dixon EP, Smalstig EB, Mincy RE, Rash KS, Little SP (1997) Global cerebral ischemia activates nuclear factor-kappa B prior to evidence of DNA fragmentation. Brain Res Mol Brain Res 48:187-196.

Deutsch DG, Lin S, Hill WA, Morse KL, Salehani D, Arreaza G, Omeir RL, Makriyannis A. Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. Biochem Biophys Res Commun. 1997 231:217-21.

Gerdeman GL, Ronesi J, Lovinger DM. (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. Nat Neurosci 5:446-451.

Gifford AN, Bruneus M, Lin S, Goutopoulos A, Makriyannis A, Volkow ND, Gatley SJ. (1999) Potentiation of the action of anandamide on hippocampal slices by the fatty acid amide hydrolase inhibitor, palmitylsulphonyl fluoride (AM 374). Eur J Pharmacol 383(1):9-14.

Giuffrida A, Beltramo M, Piomelli D. Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. J Pharmacol Exp Ther. 2001 298:7-14.

Glazner GW, Mattson MP (2000) Differential effects of BDNF, ADNF9, and TNFalpha on levels of NMDA receptor subunits, calcium homeostasis, and neuronal vulnerability to excitotoxicity. Exp Neurol 161:442-452

Goodman Y, Mattson MP (1996) Ceramide protects hippocampal neurons against excitotoxic and oxidative insults, and amyloid beta-peptide toxicity. J Neurochem 66:869-872.

Gonzalez-Garcia M, Garcia I, Ding L, O'Shea S, Boise LH, Thompson CB, Nunez G (1995) bcl-x is Expressed in Embryonic and Postnatal Neural Tissues and Functions to Prevent Neuronal Cell Death. PNAS 92:4304-4308.

Granger, R., Deadwlyer, S., Davis, M., Moskovitz, B., Kessler, M., Rogers, G., and Lynch, G. (1996). Facilitation of glutamate receptors reverses age-associated memory impairment in rats. Synapse 22, 332-337.

Grilli M, Pizzi M, Memo M, Spano P (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. Science 274:1383-1385.

Hampson, R. E., Rogers, G., Lynch, G., and Deadwyler, S. A. (1998). Facilitative effects of the ampakine CX516 on short-term memory in rats: correlations with hippocampal neuronal activity. J. Neurosci. 18, 2748-2763.

Harry, G. J., Goodrum, J. F., Krigman, M. R., and Morell, P. (1985). The use of synapsin I as a biochemical marker for neuronal damage by trimethyltin. Brain Res.326, 9-18.

Hayashi T, Umemori H, Mishina M, Yamamoto T. (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. Nature 397(6714):72-76.

Hewett SJ, Uliasz TF, Vidwans AS, and Hewett JA (2000) Cyclooxygenase-2 Contributes to N-Methyl-D-aspartate-Mediated Neuronal Cell Death in Primary Cortical Cell Culture. J Pharmacol Exp Ther 293(2), 417-425.

Koistinaho J, Koponen S, Chan PH, and Hsu CY (1999) Expression of Cyclooxygenase-2 mRNA After Global Ischemia Is Regulated by AMPA Receptors and Glucocorticoids. Stroke 30, 1900-1906.

Lauterborn JC, Lynch G, Vanderklish P, Arai A, Gall C 2000 Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. J Neurosci 20:8-21

Lebrun, C., Pilliere, E., and Lestage, P. (2000). Effects of S 18986-1, a novel cognitive enhancer, on memory performances in an object recognition task in rats. Eur. J. Pharmacol. 401, 205-212.

Limatola C, Ciotti MT, Mercanti D, Santoni A, Eusebi F. (2002) Signaling pathways activated by chemokine receptor CXCR2 and AMPA-type glutamate receptors and involvement in granule cells survival. J Neuroimmunol 123(1-2):9-17.

Lipsky RH, Xu K, Zhu D, Kelly C, Terhakopian A, Novelli A, Marini AM (2001) Nuclear factor kappaB is a critical determinant in N-methyl-D-aspartate receptor-mediated neuroprotection. J Neurochem 78:254-64.

Mattson MP (1997) Neuroprotective signal transduction: relevance to stroke. Neurosci Biobehav Rev 21(2), 193-206

Mattson, MP, C Culmsee, Y ZaiFang, and S. Camandola. 2000. Roles of nuclear factor □□□in neuronal survival and plasticity. J. Neurochem. 74: 443-456.

McKinney, R. A., M. Capogna, R. Durr, B. H. Gahwiler, and S. M. Thompson. 1999. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat. Neurosci. 2: 44-49.

Mitin N, Kudla AJ, Konieczny SF, Taparowsky EJ (2001) Differential effects of Ras signaling through NF-kappaB on skeletal myogenesis. Oncogene 20:1276-1286.

Munirathinam S, Rogers G, and Bahr B.A. (2002) Positive modulation of ampa-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus *Toxicology and Applied Pharmacology* in press

Nakai M, Qin ZH, Chen JF, Wang Y, Chase TN (2000) Kainic acid-induced apoptosis in rat striatum is associated with nuclear factor-kappaB activation. J Neurochem 74:647-58.

Nellgard B and Wieloch T. (1992) Cerebral protection by AMPA- and NMDA-receptor antagonists administered after severe insulin-induced hypoglycemia. *Exp Brain Res* 92(2):259-266

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619.

Pahl HL (1999) Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18:6853-66.

Perkins ND (2000) The Rel/NF-kappa B family: friend and foe. Trends Biochem Sci 25:434-440.

Qin ZH, Wang Y, Nakai M, and Chase TN (1998) Nuclear Factor-kappa B Contributes to Excitotoxin-Induced Apoptosis in Rat Striatum. Mol Pharmacol 53(1), 33-42.

Rakhit RD, Kabir AN, Mockridge JW, Saurin A, Marber MS (2001) Role of G proteins and modulation of p38 MAPK activation in the protection by nitric oxide against ischemia-reoxygenation injury. Biochem Biophys Res Commun 286:995-1002.

Rosenberger J, Petrovics G, Buzas B (2001) Oxidative stress induces proorphanin FQ and proenkephalin gene expression in astrocytes through p38- and ERK-MAP kinases and NF-kappaB. J Neurochem 79:35-44.

Sheardown MJ, PD Suzdak, L Nordholm 1993. AMPA but not NMDA receptor antagonism is protective in gerbil global ischemia, even when delayed 24 h. *Eur. J. Pharmacol.* **236**: 347-353.

Sonenshein GE (1997) Rel/NF-kappa B transcription factors and the control of apoptosis. Semin Cancer Biol 8:113-119.

Staubli U, Ambros-Ingerson J, Lynch G. (1992) Receptor changes and LTP: an analysis using aniracetam, a drug that reversibly modifies glutamate (AMPA) receptors. Hippocampus 2(1):49-57.

Staubli, U., Rogers, G. and Lynch, G. (1994). Facilitation of glutamate receptors enhances memory. Proc. Natl. Acad. Sci. U.S.A. 91, 777-781

Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, and Vodyanoy V (2001) Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. *Synapse* (NY) **40**:154-158.

Tamatani M, Mitsuda N, Matsuzaki H, Okado H, Miyake S, Vitek MP, Yamaguchi A, Tohyama M (2000) A pathway of neuronal apoptosis induced by hypoxia/reoxygenation: roles of nuclear factor-kappaB and Bcl-2. J Neurochem 75:683-693.

Tang CM, Shi QY, Katchman A, Lynch G. (1991) Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. Science 254(5029):288-90

Vanderklish, P. W. and B. A. Bahr. 2000. The pathogenic activation of calpain: A marker and mediator or cellular toxicity and disease states. *Internatl. J. Exp. Pathol.* **81**: 323-339.

Wang Y, Durkin JP (1995) alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but not N-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein beta gamma subunits in rat cortical neurons. *J Biol Chem* 270(39):22783-22787.

10. APPENDICES

□ MANUSCRIPTS (attached at the end)

1. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, and Rogers G (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp Neurol* **174**:37-47.

2. Caba E, Brown QB, Kawasaki B, and Bahr BA (2002) Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. *J Neuroscience Res* **67**:787-794.

3. Bendiske J, Caba E, Brown QB, and Bahr BA (2002) Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J Neuropathol Exp Neurol* **61**:640-650.

4. Vicente JS, Munirathinam S, and Bahr BA (2002) Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age. *J Mol Biol Biotech* **3**:1-9.

5. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of AMPA-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol*, in press.

6. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem*, in press.

□ ABSTRACTS (pages 25-35)

1. Caba E and Bahr BA, Biphasic response of NF-κB to stroke-type pathogenesis in organotypic hippocampal slice cultures, *GRASP Pharmacy Research Conference* (2001).

2. Bendiske J and Bahr BA, Characterization and slowing of the protein deposition and synaptic decay cascade in hippocampal slice cultures, *Second Neurobiology Conference: Identifying Accelerators and Brakes of Brain Aging* (2001).

3. Caba E, Giardina C, Rogers G, Bahr BA, Ampakine effects indicate that the biphasic NF-κB response to excitotoxicity denotes two opposing signaling pathways, *Soc. Neurosci. Abstr.*, **27**, 867.2 (2001).

4. Suppiramaniam V, Subramaniam T, Kanju PM, Kleene R, Moss A, Premkumar L, Brown Q, Bahr BA, and Dityatev A, Single channel recordings from mossy fiber synaptosomes: Evidence for the presence of high-affinity synaptic AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 502.10 (2001).

5. Subramaniam T, Kleene R, Manivannan K, Dityatev A, Bahr BA, and Suppiramaniam V, Polysialic acid modulates single channel properties of synaptosomal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 610.5 (2001).

6. Chicoine L, Suppiramaniam V, Gianutsos G, Kawasaki BT, and Bahr BA, Sulfated polysaccharides modulate calcium permeability and binding properties of hippocampal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 480.6 (2001).

7. Bahr BA, Bendiske J, Michaelis ML, Georg G, Caba E, and Brown Q, Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders, *Soc. Neurosci. Abstr.*, **27**, 581.2 (2001).

8. Bendiske J and Bahr BA, Positive lysosomal modulation reverses the PHF-tau accumulation ◇ microtubule destabilization ◇ transport failure ◇ synaptic deterioration cascade, *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001).

9. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, The majority of cell death occurs early after intracerebral grafting of ventral mesencephalic tissue: A role for calpain activation. *Soc. Neurosci. Abstr.*, **27**, 197.4 (2001).

10. Bahr BA, Caba E, Giardina C, Brown QB, and Bendiske J, Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways, *Mol. Biol. Cell*, **12** (Suppl.), 2233 (2001).

11. Munirathinam S and Bahr BA, Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *The Toxicologist* (Society of Toxicology), **58**, 1551 (2002).

❏ CURRICULUM VITAE: Ben A. Bahr (pages 36-56)

11. PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Ben A. Bahr, Ph.D., P.I.

Subramani Munirathinam, Ph.D., postdoctoral researcher

Queenie Brown, M.S., research technician

Ebru Caba, M.S., graduate student/research assistant

Jen Bendiske, B.S., Ph.D., graduate student/research assistant

David Karanian, B.S., graduate student/research assistant

1. <u>Caba E</u>, and Bahr B.A. Biphasic Response Of NF-κB To Stroke-Type Pathogenesis In Organotypic Hippocampal Slice Cultures. *GRASP Pharmacy Research Conference (2001)*.

Purpose: To investigate the possible involvement of signaling elements in an excitotoxic event. To characterize the two phases of NF-κB activation in a model of stroke pathogenesis. **Methods**: Organotypic hippocampal slice cultures were prepared from 12 day old Sprague-Dawley rats. 400 μm transverse sections were prepared and maintained under humidified conditions with 5% CO_2 at 37°C for 15-20 days before use. Slices were exposed to 200 μM NMDA, followed by quenching with NMDA and AMPA receptor antagonists and maintained in culture for 1-24 h. Ampakine modulation was used in the incubation step to elucidate the mechanism of neuroprotection provided. **Results**: The resulting NF-κB activation exhibited a biphasic response profile (ANOVA: $p<0.0001$) consisting of a rapid 4-fold increase ($p<0.001$, post-hoc test), a subsequent decline by 36% after 5 h ($p<0.01$), and a delayed increase. The latter represented a 6-fold rise from control levels by 24 h post-insult ($p<0.001$), and a 53% increase above the peak of the first phase. A neuroprotectant was used to characterize the biphasic response and to address whether the biphasic nature explains NF-κB's dual role in cell signaling. A potent Ampakine derivative shown to be neuroprotective through the MAP kinase pathway was applied to slices at 10-30 μM, causing a progressive increase in NF-κB activity over 24 h. After an NMDA insult, however, Ampakine modulation produced only a small change in NF-κB activity measured 0.5-2 h post-insult (NS). **Conclusions**: These results suggest that the early NF-κB response is involved in protection since the extent of activation was not broadened by the neuroprotectant. These data provide evidence that the early NFκB response to an excitotoxic episode is primarily acting as a protective compensatory mechanism, as indicated by the inability of a neuroprotectant to contribute additively. Conversely, a second phase of NFκB activation occurring late after the excitotoxic event appears to be pathogenic, perhaps proinflammatory. The latter is indicated by the fact that the delayed NFκB response to the excitotoxin is acting through a separate mechanism from that produced by the neuroprotectant.
Support: Boehringer Ingelheim, NIH grant 1R43NS38404-01.

2. Bendiske J and Bahr BA, Characterization and slowing of the protein deposition and synaptic decay cascade in hippocampal slice cultures, *Second Neurobiology Conference: Identifying Accelerators and Brakes of Brain Aging* (2001).

Protein deposition in AD is associated with enhanced levels of lysosomal enzymes. Activation of the lysosomal system may be a compensatory response against protein deposits. In hippocampal slice cultures, abnormal processing mediated by the lysosomal disruptor chloroquine (Cqn) led to tau and amyloidogenic deposits, microtubule destabilization, transport failure, and synaptic decline. As this cascade progressed, increases in cathepsin isoforms, elastase and β-glucuronidase occurred. To test whether this lysosomal activation represents a compensatory response, Z-Phe-Ala-diazomethylketone (PADK) was identified as a positive modulator at low levels, generating robust up-regulation of lysosomal enzymes (400-600%) over that which occurred in response to Cqn (70-100%). PADK-mediated modulation was stable for >20 days while synaptic protein levels remained normal. When PADK and Cqn were co-infused, Cqn no longer elevated 55-69kDa tau levels. To assess pre-existing conditions, Cqn was applied for 6 days after which removing the agent resulted in continued pathogenesis. Replacing Cqn with PADK after the 6 days led to reduced deposition of PHF material and APP fragments. Less deposits was linked to restoration of acetylated tubulin, a marker of stable microtubules. Transport processes lost during Cqn exposure also were restored by PADK, resulting in up to 70% recovery in expression of synaptic markers. These data indicate that lysosomal modulation can enhance cellular repair potential.

3. Caba E, Giardina C, Rogers G, Bahr BA, Ampakine effects indicate that the biphasic NF-κB response to excitotoxicity denotes two opposing signaling pathways, *Soc. Neurosci. Abstr.*, **27**, 867.2 (2001).

NF-κB activity was evaluated in hippocampal slice cultures after stroke-type excitotoxicity. Slices were exposed to 200 μM NMDA for 20 min, followed by quenching with antagonists. The resulting NF-κB activation exhibited a biphasic response profile (ANOVA: $p < 0.0001$) consisting of a rapid 4-fold increase ($p < 0.001$, post-hoc test), a subsequent decline by 36% after 5 h ($p < 0.01$), and a delayed increase. The latter represented a 6-fold rise from control levels by 24 h post-insult ($p < 0.001$), and a 53% increase above the peak of the first phase. A neuroprotectant was used to characterize the biphasic response and to address whether the biphasic nature explains NF-κB's dual role in cell signaling. A potent Ampakine derivative shown to be neuroprotective through the MAP kinase pathway was applied to slices at 10-30 μM, causing a progressive increase in NF-κB activity over 24 h. After an NMDA insult, however, Ampakine modulation produced only a small change in NF-κB activity measured 0.5-2 h post-insult (NS). This suggests the early NF-κB response is involved in protection since the extent of activation was not broadened by the neuroprotectant. In contrast, the Ampakine effect was additive during NF-κB's delayed response to the insult (+107%, $p < 0.01$). This indicates that Ampakine neuroprotection and the second phase of NF-κB activation are mediated through two separate mechanisms; the delayed NF-κB response perhaps is pathogenic. The biphasic nature of its activation may explain why NF-κB is implicated in both survival and apoptosis (support: Boehringer Ingelheim, U.S. Army grant DAMD17-99-C9090; NIH grant 1R43NS38404-01).

4. Suppiramaniam V, Subramaniam T, Kanju PM, Kleene R, Moss A, Premkumar L, Brown Q, Bahr BA, and Diyatev A, Single channel recordings from mossy fiber synaptosomes: Evidendce for the presence of high-affinity synaptic AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 502.10 (2001)

AMPA receptors are primary mediators of fast excitatory neurotransmission. Previous studies have demonstrated the existence of low and high affinity AMPA receptors and the former being widely present in synaptic plasma membrane. Our preliminary data indicates the presence of high affinity AMPA receptors in murine mossy fiber synaptosomes. We have investigated the single channel properties of AMPA receptors by incorporating synaptosomes into artificial lipid bilayers. Our study indicates that in the presence of Na^+,K^+ and NMDA antagonists 150 nM of AMPA was sufficient to elicit single channel currents in most (96 %) experiments. These currents were sensitive to 1M CNQX. Thus our data indicate the existence of high affinity AMPA receptors in synaptosomes, i.e. synaptically and/or perisynaptically. The two major conductance states of 10 pS and 50 pS that were observed for the synaptosomal AMPA receptors are similar to those observed with purified AMPA channels. Further studies are conducted to confirm the existence of varying conformational states of these receptors in their native environment. *Supported by: NIH/NINDS*

5. Subramaniam T, Kleene R, Manivannan K, Dityatev A, Bahr BA, and Suppiramaniam V, Polysialic acid modulates single channel properties of synaptosomal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 610.5 (2001).

Polysialic acid (PSA), a homopolymer of sialic acid decorating the neural cell adhesion molecule (NCAM) is essential for long lasting changes in synaptic efficacy. Our previous studies indicate that PSA altered the channel properties of purified AMPA receptors reconstituted in artificial lipid bilayer. This study aimed to demonstrate the modulation of synaptic AMPA receptors by PSA. We have utilized isolated murine mossy fiber synaptosomes incorporated into lipid bilayers to analyze the single channel properties of synaptosomal AMPA receptors. Channel recordings were made in the presence of 150 nM of AMPA. Addition of colominic acid (bacterially produced PSA) prolonged the single channel open times in a dose-dependent manner. Burst analysis revealed that PSA decreased the inter-burst interval and increased the mean burst duration and duration of openings within the burst without altering the single channel conductance. The above findings suggest that PSA, which is expressed in synapses, in activity-dependent manner may influence synaptic strengthening by modulating AMPA channel properties. *Supported by: NIH/NINDS*

6. Chicoine L, Suppiramaniam V, Gianutsos G, Kawasaki BT, and Bahr BA, Sulfated polysaccharides modulate calcium permeability and binding properties of hippocampal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 480.6 (2001).

AMPA-type glutamate receptors have been shown to bind heparin, resulting in a marked reduction in AMPA binding affinity and a corresponding increase in the open channel lifetime. In the present study, dextran sulfate was found to be more potent than heparin in altering binding and channel properties. Further studies were conducted to assess changes in calcium permeability in primary hippocampal neurons. Calcium-activated fluorescent dyes were loaded into neurons, and calcium influx was specifically induced by stimulating AMPA receptors. Dextran sulfate (500 kDa) enhanced the AMPA-induced calcium influx by as much as 50%, and did so in a similar dose-dependent manner as that found for changes in binding properties (EC50s: 10 nM). Confocal imaging showed that the increase in calcium permeability occurred in hippocampal neuronal cell bodies and their processes, and the increase was evident at concentrations of dextran sulfate as low as 0.1 nM. The effects of dextran sulfate were blocked by CNQX but not by the NMDA receptor antagonist AP5. The 500 kDa dextran sulfate was much more effective than smaller forms of the polysaccharide, and non-sulfated dextran was completely ineffective at altering AMPA receptor binding and channel activities. Together, these findings suggest that large, sulfated polysaccharides can modulate AMPA receptor properties in hippocampal neurons. *Supported by: NIH grant #IR43NS38404-01*

7. Bahr BA, Bendiske J, Michaelis ML, Georg G, Caba E, and Brown Q, Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders, *Soc. Neurosci. Abstr.*, **27**, 581.2 (2001).

Injury-induced compensatory responses and neuroprotection were studied in brain slice cultures. In response to excitotoxicity, MAPK was activated through MEK. We identified a link between AMPA receptors and MAPK, and found that it functions to reduce excitotoxic risk. Blocking the AMPA receptor-MAPK pathway enhanced cytoskeletal vulnerability and NF-κB activation, two responses to excitotoxicity. Correspondingly, positive modulation of the pathway enhanced recovery from excitotoxic injury. For age-related studies, lysosomal inhibition was induced over 8 days, revealing a connection between synaptic decay and abnormal processing of APP and tau. Additional correspondences to AD-type events occurred including microtubule destabilization, transport failure, and up-regulation of cathepsin D. The latter compensatory response was induced with a positive lysosomal modulator in slices with perturbed lysosomes, resulting in enhanced tau processing, microtubule stability markers (acetyltubulin: Ac-TN), and synaptic recovery; such protection was not induced by the AMPA receptor-MAPK pathway. To determine the role of microtubule stabilization, the Taxol derivative Tx50 was applied to compromised slices causing an 82% recovery in Ac-TN. Interestingly, Ac-TN correlated with synaptic markers which increased 2-fold. When Tx50 was added before synaptic decay, lysosomal dysfunction had no effect. Thus, distinct compensatory responses underlie the brain's capacity for self-repair, and microtubule-based transport mechanisms are particularly targeted by the synaptopathogenic cascade. Support: DOD DAMD17-99-C9090, NIH 1R43NS38404-01.

8. Bendiske J and Bahr BA, Positive lysosomal modulation reverses the PHF-tau accumulation ◇ microtubule destabilization ◇ transport failure ◇ synaptic deterioration cascade, *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001).

Long-term hippocampal slices were used to define the sequence of, and possible interactions between, the changes associated with age-related diseases. Such studies previously identified a link between abnormal protein processing and synaptic deterioration, two major correlates observed in Alzheimers. In addition to amyloidogenic changes, lysosomal dysfunction induced a distinct series of alterations: 1) increased levels of phosphorylated tau (p-tau) and aggregates recognized by antibodies to human PHF-tau, 2) reduced levels of acetylated tubulin - a marker of stable microtubules (MTs), and 3) loss of transport capability. To test if such a cascade is indeed responsible for the loss of synaptic proteins, we utilized positive lysosomal modulation. When a modulator was applied to slices expressing all steps of the noted cascade, there was a 4-6 fold increase in cathepsin D, a lysosomal enzyme whose action may explain the corresponding decline in p-tau levels. The enhanced lysosomal capacity also caused the diminished levels of acetylated tubulin to be increased 2-3 fold, suggesting that MT stabilization was positively affected. Finally, pre- and postsynaptic markers indicated that lysosomal modulation reversed the pathogenic cascade, resulting in a 71% recovery ($p < 0.0001$). In control slices, lysosomal modulation increased cathepsin D levels without changing any of the other parameters. The identified cascade is confirmed by the correlative events evident during both the pathogenic and protective directions. These data indicate that early stages of age-related disorders can be treated by targeting the initial step in the synaptopathogenic cascade.

9. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, The majority of cell death occurs early after intracerebral grafting of ventral mesencephalic tissue: A role for calpain activation. *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001)

Neural transplantation is an experimental treatment for Parkinson's disease, however, only around 10% of the implanted neurons survive the procedure. Earlier studies have indicated that a large proportion of the grafted cells die during graft tissue preparation and within the first few days after intracerebral implantation. The present study was designed to reveal cell death in intrastriatal grafts, starting at 90 minutes after implantation, compared to the prevalence of cell death after 1, 3, 6 and 42 days. We identified abundant degenerating cell profiles soon after grafting using Nissl stained semithin sections. We used a hairpin probe to detect specific DNA breaks and antibodies against caspase-3 to monitor apoptotic cell death. In addition, we studied calpain activation in grafted cells and host striatum using immunohistochemistry for a calpain-specific fodrin breakdown product. The results indicate that there is a marked calpain activity already at 90 minutes and that the majority of the implanted cells die within 6 days after grafting. Therefore, we speculate that several different mechanisms of cell death may occur in ventral mesencephalic transplants. This implies that future efforts to further enhance the survival of grafted dopamine neurons should not only focus on anti-apoptotic strategies but also consider the use of calpain inhibitors to counteract other types of cell death.

10. Bahr BA, Caba E, Giardina C, Brown QB, and Bendiske J, Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways, *Mol. Biol. Cell*, **12** (Suppl.), 2233 (2001).

Following a 24-h recovery period, a further increase of 73% ($p<0.01$; n=13) was observed, suggesting a biphasic nature in NF kB's response during post-insult recovery. In an attempt to discriminate between protective and proinflammatory response components, additive effects were tested with a potent Ampakine (Cortex Pharm.), a neuroprotectant that appears to act through MAP kinase pathways to reduce excitotoxin-induced cytoskeletal damage ($p<0.01$) and pre- and postsynaptic decay. Ampakine alone (10-20 μM) indeed caused an approximate 2-fold increase in NFkB activation after 1-5 h incubation ($p=0.02$), and a 6-fold increase after 24 h ($p<0.0001$). When applied after the 20-min NMDA treatment, however, Ampakine only produced a small additive effect on NFkB activity at 1-5 h post-insult (31 ± 20%) which was not significant. Interestingly, Ampakine did have an additive influence on the NMDA-mediated response (108 ± 41%, $p=0.01$; n=13) at 24 h post-insult. These data provide evidence that the early NFkB response to an excitotoxic episode is primarily acting as a protective compensatory mechanism, as indicated by the inability of a neuroprotectant to contribute additively. Conversely, a second phase of NFkB activation occurring late after the excitotoxic event appears to be pathogenic, perhaps proinflammatory. The latter is indicated by the fact that the delayed response to the excitotoxin is clearly acting through a separate mechanism from that produced by the neuroprotectant Ampakine (USAMRMC grant DAMD17-99-C9090, NIH grant 1R43NS38404-01).

11. Munirathinam S and Bahr BA, Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *The Toxicologist* (Society of Toxicology), **58**, 1551 (2002).

Hippocampal slice cultures were used to examine the action of the organophosphate agent soman (GD). Soman is known to inhibit acetylcholinesterase, causing accumulation of ACh, but it also appears to increase glutamate release to promote seizure-related damage. In the present study, the effects of low concentrations of soman on hippocampal tissue were assessed over an extended series of exposures. Single application was used to induce a period of toxicity until agent hydrolysis, and this was repeated daily for one week. No evidence of degeneration was found after the first day of treatment, however, dose-dependent declines in pre- and postsynaptic proteins occurred after a week. The decline in synaptophysin was evident at the 100-nM dose of soman, and further reduction was found at 20 µM (ANOVA: $p=0.001$, $F=9.7$). Synaptophysin was reduced throughout the hippocampus, especially in dendritic fields of CA1 and in the molecular layer of the dentate gyrus. Synaptic toxicity appears first since overt cellular pathology was not visible in the three subfields. The decline in the postsynaptic GluR1 subunit was only evident at the 20-µM soman dose ($p<0.001$, $F=11.8$). Soman also elicited a pronounced increase ($p<0.01$) in spectrin breakdown mediated by calpain, a protease known to cause neurodegeneration in response to glutamate-induced excitotoxicity. Interestingly, while no degeneration was found after the first day of soman treatment, pathogenic changes did occur when the soman insult was combined with a trimethyltin (TMT) insult - TMT is known to cause seizures and hippocampal damage. A 1-h TMT exposure did not cause appreciable synaptic deterioration or spectrin breakdown in control slices, however, robust levels of degeneration were evident when such a TMT insult was followed by a single application of soman. Thus, low-level soman exposure causes synaptic damage in the hippocampus and potentiates vulnerability to other types of toxic insults. Supported by U.S. Army Med. Res. grant DAMD17-99-C9090, and NIH grant 1R43NS38404-01.

❑ CURRICULUM VITAE: Ben A. Bahr (pages 36-56)

Associate Professor of Pharmacology and Neurotoxicology
Department of Pharmaceutical Sciences and the Neurosciences Program
Center for Drug Discovery
University of Connecticut, Storrs, Connecticut 06269-2092
Bahr@uconn.edu   tel 860-486-6043   fax 860-486-6858

DEGREES

1984; BA (Molecular Biology) Dept. of Biological Sciences, University of California, Santa Barbara
1984; BA (Biochemistry)   Dept. of Biological Sciences, University of California, Santa Barbara
1989; PhD (Chemistry)   Dept. of Chemistry, University of California, Santa Barbara

POSITIONS

2001 -	Associate Professor (tenured), Dept. of Pharm. Sciences, University of Connecticut
1997-01	Assistant Professor, Dept. of Pharmaceutical Sciences, University of Connecticut
1995-96	Nontenure-track faculty (Associate), Ctr Neurobiol Learning/Memory, Univ. of Calif.
1991-94	Nontenure-track faculty (Assistant), Ctr Neurobiol Learning/Memory, Univ. of Calif.
1990-94	Lecturer, Dept. Psychobiol. and Ctr Neurobiol Learning/Memory, Univ of Calif, Irvine
1989-91	NRSA Postdoctoral Fellow, Ctr Neurobiol Learning/Memory, Univ. of Calif., Irvine
1985-89	Predoctoral Fellow, Dept. of Chemistry and the Neuroscience Institute, Univ. of Calif.

Consultantships:

2001 -	Consultant/Collaborator, Bristol-Myers Squibb, Wallingford, Connecticut
2001 -	Consultant/Collaborator, Merz Pharmaceuticals, Inc., Frankfurt, Germany
1999 -	Consultant, Cognetix, Inc., Ivorytown, Connecticut
1996 -	Consultant/Collaborator, Cortex Pharmaceuticals, Inc., Irvine, California

DISTINCTIONS

The Professor B.R. Baker Memorial Award in Chemistry, 1987

The American Chemical Society Pacific Division Invited Keynote Address, 1991

Young Investigator Award from the University of California, Irvine, 1995

Faculty Career Development Award from the University of California, Irvine, 1996

Colloquium Organizer and Chair for the American Society for Neurochemistry, 2000

Young Investigator Award from the International Society for Neurochemistry, 2001

RESEARCH INTERESTS

♦ Links Between Receptor Signaling Events, Synaptic Regulation, and Cellular Repair
♦ Neuroprotection in Models of Age-Related Diseases, Stroke, and Neurotoxin Exposure
♦ Pathogenic Cascades Involving Aberrant Protein Processing and Synaptic Failure

PATENTS

1999   Brain Aging Assay (U.S. Patent Application WO1998US0001140)

2000   Positive Lysosomal Modulation and Use for Treating Neurodegeneration (U.S. 60/244,327)

PROFESSIONAL AFFILIATIONS

Society for Neuroscience, Member since 1985

American Society for Cell Biology, Member since 1990

American Society for Pharmacology & Experimental Therapeutics, Member since 1995

Society of Toxicology, Member since 1998

American Society for Neurochemistry, Member since 1998

Proteome Society, Member since 2001

PROFESSIONAL ACTIVITIES

1989-93	Organization Committee Member, Human Frontiers Science Program, Univ. of Calif.
1990-91	Member of the Health Sciences Advisory Committee, Univ. of Calif., Irvine
1990-92	Chair of the Hebb Seminars, Ctr. Neurobio. Learning/Memory, Univ. of Calif., Irvine
1990-92	Research Advisor: The Gerard Award Program, Dept. of Psychobiol., Univ. of Calif.
1990-92	Member of the Seminar and Workshop Program Committee, Univ. of Calif., Irvine
1991	NSF/NATO/FEBS International Travel Award, symposium presenter
1992-93	Research Advisor: The Dean's Res. Award Program, College of Med., Univ. of Calif.
1992-95	Organization Committee Member, Irvine Institute for Brain Aging & Dementia
1992-93	Research Advisor: NSF Undergrad. Research Program in Psychobiol., Univ. of Calif.
1992-94	Advisor for President's Undergrad. Res. Fellowship, Sch. of Biol. Sci., Univ. of Calif.
1993	Research Proposal Reviewer, NSF Program of Neuroscience
1993-	Peer Review Consultant, National Institutes of Health / ADAMHA
1993-94	Research Advisor: Instructional Development Fellowship Program, Univ. of Calif.
1995-97	Reviewer for *J Neuroscience, J Neurobiology of Learning and Memory, Hippocampus*
1996-97	Chair, Institutional Animal Care and Use Committee, Cortex Pharmaceuticals
1998-01	Member of the Pharmacology Faculty Search Committees, University of Connecticut
1998-01	Program Member for Tuskegee-Auburn Universities Neuroscience Conference Series
1998-	Reviewer for *J Neuroscience, J Comparative Neurology, J Neurochemistry, Mol Brain Research, Neuroscience*
1999-00	Symposium Program, American Society for Neurochemistry Meeting
1999-01	Secretary of the School of Pharmacy Faculty, University of Connecticut
2000	Organizer for the University of Connecticut School of Pharmacy Hewitt Symposium
2000	Colloquium organizer/chair for American Society for Neurochemistry annual meeting
2000-	Review board member for the *Journal of Molecular Biology and Biotechnology*

2000-	Officer of Animal Care and Safety, School of Pharmacy, University of Connecticut
2001-	Member of the Neurosciences Program Steering Committee, University of Connecticut
2001	Reviewer for the Alzheimer's Association's 2001 Grants Program
2001-	Consultant for the Virginia Commonwealth Neurotrauma Initiative Grant
2001	Newspaper interview: Associated Press (health segment on memory overload).
2001-02	Mentor for UConn Neurosciences Graduate Student Fellow
2001-	Mentor of Boehringer-Ingelheim Graduate Student Fellow
2001	Honorary membership to the Australian Neuroscience Society, presenter
2001-	Consultant, Univ. of Connecticut-Hartford Hospital Huntington's Disease Program
2001-	Pharm/Tox Representative for the Dept. Pharmaceutical Sci. Steering Committee
2001-03	Undergraduate Research Advisory Committee member (2-year term), Univ. of Conn.
2001	UConn Advance newspaper interview: New treatment strategy for Alzheimer's disease.
2002	Reviewer of MedChem dissertation prospectus, Center for Drug Discovery, UConn
2002	Reviewer for Research Foundation Internal Awards Program, Univ. of Connecticut
2002	UConn Tradition magazine interview: How to study neurological disorders.
2002	Reviewer for professor DSc promotion, University of London
2002	Television interview: Channel 30 News (self-repair systems in the brain).

TEACHING

Undergraduate Student Education, University of California, Irvine

Neural Systems - Mechanisms of Brain Function	1990-94
Biology of Behavioral Disorders	1991

Mentoring, University of California, Irvine

Undergraduate Researchers:

Peter Vanderklish		1989-92
Mo Tin		1989-91
Lu Ha		1989-91
Henry Ta		1990-92
Barbara Bakus	(Dean's Res. Award Program fellow)	1990-92
Dino Capaldi		1990-93
Sheri Viteri		1990-93
Nam Lam		1991-93
Babak Abai	(NSF Undergrad. Res. Program fellow)	1991-94
Steve Shahrestani		1991-93
D. Tyler McQuade	(President's Undergrad. Res. Fellowship)	1991-94
Annette Godshall		1991-94
Everard Esteban	(Gerard Award fellow)	1991-94
Ramon Rosario		1992-94
Joycelyne Vicente		1993-96
Jason Sharp		1993-95
Keith Hoffman	(Instructional Development Fellowship)	1993-95
Suma Tiriveedhi		1993-96
George Park		1994-96
Manpreet Singh (Mutneja)		1994-96
Brian Kawasaki		1994-97
Tu Pham		1995-96

Co-Advisor for Graduate Students:

Randy Hall	1991-95
Peter Vanderklish	1992-93
Keith Hoffman	1995-98
Eric Bednarski	1995-96

Postdoctoral Co-Mentorship:

Dr. Peng Xiao	1991-92

TEACHING (continued)

<u>University of Connecticut Pharm.D. Program</u>

Neuropharmacology	1998 -
Biostatistics / Literature Evaluation Skills	1999 -
Neurotoxicology Workshop	1999 -
General principles/organ systems (membrane transport)	2001

<u>Graduate Student Education, University of Connecticut</u>

Advanced Pharmacology	1998 -
Special Topics in Pharmacology/Neuroscience	1998 -
Synaptic Plasticity (Dept. of Psychology)	1999
Memory Disorders Discussion Lab	1999 -
Advanced Toxicology (pesticides/excitotoxicity)	2001
Function and Dysfunction of Brian Synapses	2001 -

<u>Mentoring, University of Connecticut</u>

Major Advisor:

Kevin Quackenbush	(Pharmacology)	1998
Queenie Brown	(Pharmacology)	1999
Jennifer Bendiske (Neurosci. Fellow) (Ph.D., Pharm/Toxicol)		1997-01
Ebru Caba (Neurosci. Fellow) (Ph.D., Pharm/Toxicol)		1998-
Linda Chicoine	(Ph.D., Pharm/Toxicol)	1999-
David Karanian	(Ph.D., Pharmacology)	2000-
Sokhom Pin	(Ph.D., Pharmacology)	2001-
Sum Lam	(Pharm.D. Honors Thesis)	1999-01

Member, Thesis Examining Committee:

Mehmet Inan	(Ph.D., Mol. Cell Biology)	2000
Brian Carlson	(Ph.D., Psychology)	2001

Postdoctoral Mentorship:

Dr. Subramani Munirathinam	1999-02
Dr. Paul Fitzmaurice	2002-

Research Scientists:

Queenie Brown	2000 -
Melissa Mendez	2000-02
Regina Vontell	2000-02

Undergraduate Researchers:

Ellen Chung	1999-02
Osama Abdelghany	1999-02
Ben Enos	current

Visiting Scientists:

Dr. Alison Beckmann	Yale University	1998
Dr. Yasuji Matsuoka	New York University	2000
Dr. Andrea Baude	M** Pharmaceuticals	2001

INVITED SEMINAR PRESENTATIONS

1989, University of Washington Department of Physiology and Biophysics Seminar, "Copurification of the Vesamicol Receptor and the SV1 Antigen of *Torpedo* Synaptic Vesicles: Is the Acetylcholine Transporter a Proteoglycan?", Seattle, Washington.

1989, University of California Molecular Biology and Biochemistry Section Seminar, "Neurotransmitter Storage Properties in Cholinergic Neurons", Santa Barbara, California.

1990, Academy of Sciences Institute of Neurobiology and Brain Research Seminar, "Transmembrane Linkages Between the Extracellular Matrix and the Cytoskeleton Affecting Long-Term Synaptic Modulation", Magdeburg, East Germany.

1990, Univ. of California Neuroscience Research Seminar, "Identification of Synaptosomal Adhesion Components and their Role in Neuronal Plasticity", Santa Barbara, California.

1990, Univ. of California Medical Center Seminar, "Disassembling and Reassembling Synapses: Points of Contact Between Plasticity and Pathology", City of Orange, Calif.

1991, The American Chemical Society Conference: Keynote Address for the ACS-CACT Joint Chemistry Update Meeting, "In Search for the Molecules of Memory", Anaheim, CA.

1992, Beckman Institute Neuronal-Pattern-Analysis Seminar, "Adhesion Molecules, AMPA Receptors, and Long-Term Potentiation", University of Illinois, Urbana-Champaign, Illinois.

1993, Winter Conference on Neural Plasticity, Fifth Annual Meeting: Symposium on Long-Term Potentiation - Mechanisms of Maintenance, "Involvement of Adhesion Molecules in the Functional Reorganization of Synaptic Contacts", Saint Lucia, West Indies.

1994, Univ. of California Learning and Memory Seminar, "Alternative Approach Toward the Understanding of Brain Aging: A Model that Can Express Cellular Events Prominent in the Aged Human Brain and Alzheimer's Disease in Days Instead of Decades", Irvine, California.

1995, The American Association of Retired Persons (AARP): Keynote Address for Nevada Regional Meeting, "New Strategies, Research Tools, and Memory Drugs Directed at Alzheimer's Disease and Other Related Pathologies", Reno, Nevada.

1996, Spring Hippocampus Research Conference (V), "Distinct Adhesive Chemistries and the Functional Modulation of Synapses: A Possible Connection Between Synaptic Architecture and the Stabilization of Receptor Regulation Systems", Grand Cayman, British West Indies.

1997, University of Oxford Calpain Symposium: New Therapeutics, "Calpain Activation in Long-Term Hippocampal Slices: A Model of Neurotoxicity", Oxford, United Kingdom.

1997, Auburn University Receptor Physiology Seminar: "Regulation of Glutamatergic Activity by Structural Signaling Elements, Extrasynaptic Matrix Components, and Novel Allosteric Modulators", Auburn, Alabama.

1998, University of Connecticut Department of Physiology and Neurobiology Seminar: "Cytoskeletal Reorganization Events Underlying Synaptic Plasticity and Their Potential Link to Neuroprotection", Mansfield-Storrs, Connecticut.

1999, Connecticut Center on Aging Seminar: "An Alternative for Studying Age-Related Neuropathology and Potential Treatment with New Memory Enhancing Drugs", Storrs, CT.

1999, University of Connecticut Department of Psychology: "Development of an Aging Model to Study Processes that Lead to Synaptic Pathology", Mansfield-Storrs, CT.

1999, University of Connecticut Health Center, Claude Pepper Older Americans Center Research Seminar: "Development of a Model of Age-Related Neurodegeneration for the Study of Potential Intervention Strategies", Farmington, Connecticut.

2000, Pfizer Inc., Central Research Division Invited Speaker: "Alzheimer-Type Pathogenesis and Potential Neuroprotection Avenues: Unique Ideas from a Unique System", Groton, CT.

2000, American Society for Neurochemistry Colloquium: "Multifaceted Properties of AMPA Receptors Include Links to Neuroprotective Signaling Pathways", Chicago, Illinois.

2000, Hewitt Symposium on Neurotherapeutic Research: "Advances in an *In Vitro* Model of Age-Related Neuropathogenesis", Storrs, Connecticut.

2000, Nathan Kline Institute/NYU Medical School Seminar: "Abnormal Protein Processing is Linked to Microtubule Destabilization and Synaptic Decline", Orangeburg, New York.

2001, Workshop on DoD Sponsored Parkinson's Related Research: "A Novel Protective Signaling Pathway Identified in a Model of Neurotoxin Exposure", Potomac, Maryland.

2001, Joint Asian Pacific Society for Neurochemistry/Australian Neuroscience Society, Alzheimers Pathology Symposium: "A Link Between Microtubule and Synaptic Failure in an *In Vitro* Model of Alzheimer-Type Abnormal Protein Processing," Brisbane, Australia.

2001, Joint International Society for Neurochemistry/American Society for Neurochemistry Colloquium: "Therapeutic Strategies Identified with an *In Vitro* Model of Stroke and Alzheimer's Disease", Rio de Janeiro, Brazil.

2001, International Society for Neurochemistry Colloquium: "Dynamic Structural Chemistries Underlying Synaptic Plasticity", Buenos Aires, Argentina.

2001, Pfizer Inc., CNS Program Invited Speaker: "Lypex-Induced Reversal of Alzheimer-Type Pathogenesis", Groton, Connecticut.

2002, International Conference on Alzheimer's Disease: "Activation of the Lysosomal System Reverses Neuropathogenesis in the Slice Model of Protein Deposition", Stockholm, Sweden.

PUBLICATIONS

1.   Bahr BA and Parsons SM (1986) Demonstration of a receptor for the acetylcholine storage blocker *l-trans*-2-(4-phenyl-[3,4-^{3}H]piperidino)cyclohexanol. *Proc Natl Acad Sci USA* **83**:2267-2270.

2.   Bahr BA and Parsons SM (1986) Acetylcholine transport and drug inhibition kinetics in *Torpedo* synaptic vesicles. *J Neurochem* **46**:1214-1218.

3.   Parsons SM, Anderson DC, Bahr BA, and Rogers GA (1986) A new pharmacological tool to study acetylcholine storage in nerve terminals. In Hanin I (ed): *Dynamics of Cholinergic Function*. New York, NY: Plenum Press, pp 1169-1176.

4.   Anderson DC, Bahr BA, and Parsons SM (1986) Stoichiometries of acetylcholine uptake, release, and drug inhibition in *Torpedo* synaptic vesicles. *J Neurochem* **46**:1207-1213.

5.   Parsons SM, Bahr BA, Gracz LM, Kornreich WD, and Rogers GA (1987) Uptake system for acetylcholine in isolated *Torpedo* synaptic vesicles and its pharmacology. In Dowdall MJ, Hawthorne JN (eds): *Cellular and Molecular Basis of Cholinergic Function*. Chichester, U.K.: E. Horwood Ltd., pp 303-315.

6.   Parsons SM, Bahr BA, Gracz LM, Kaufman R, Kornreich WD, Nilsson LM, and Rogers GA (1987) Acetylcholine transport: Fundamental properties and effects of pharmacologic agents. *Ann NY Acad Sci* **493**:220-233.

7.   Rogers GA, Nilsson LM, Bahr BA, Kornreich WD, and Parsons SM (1987) The most interesting members of the AH5183 family of drugs. In Dowdall MJ, Hawthorne JN (eds): *Cellular and Molecular Basis of Cholinergic Function*. Chichester, U.K.: E. Horwood Ltd., pp 333-337.

8.   Parsons SM, Rogers GA, Anderson DC, Bahr BA, Gracz LM, and Kornreich WK (1987) Inhibition of acetylcholine transport by AH5183. In Tucek S (ed): *Synaptic Transmitters and Receptors*. Prague, Czechoslovakia: J. Wiley and Sons, pp 51-54.

9.   Parsons SM, Noremberg K, Rogers GA, Gracz LM, Kornreich WD, Bahr BA, and Kaufman R (1988) Complexity and regulation in the acetylcholine storage system of synaptic vesicles. In Zimmermann H (ed): *NATO ASI Series: Cellular and Molecular Basis of Neuronal Signalling (Synaptic Transmission)*. Berlin, FRG: Springer-Verlag, vol. H21, pp 325-335.

10.   Bahr BA (1989) *The Acetylcholine Storage System and Drug Inhibition in Cholinergic Synaptic Vesicles*. Ann Arbor, MI: University Microfilms, Inc., 221 pp.

11.   Rogers GA, Parsons SM, Anderson DC, Nilsson LM, Bahr BA, Kornreich WD, Kaufman R, Jacobs RS, and Kirtman B (1989) Synthesis, *in vitro* acetylcholine-storage-blocking activities, and biological properties of derivatives and analogues of *trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol). *J Med Chem* **32**:1217-1230.

12.   Bahr BA and Parsons SM (1989) The synaptic vesicle vesamicol (AH5183) receptor contains a low affinity acetylcholine binding site. In Snoek G (ed): *NATO ASI Series: Receptors, Membrane Transport, and Signal Transduction*. Heidelberg, FRG: Springer-Verlag, vol. H29, pp 233-241.

13.   Bahr BA, Sheppard A, and Lynch G (1991) Fibronectin binding by brain synaptosomal membranes may not involve conventional integrins. *NeuroReport* **2**:13-16.

14.   Sheppard A, Wu J, Bahr BA, and Lynch G (1991) Compartmentation and glycoprotein substrates of calpain in the developing rat brain. *Synapse* (NY) **9**:231-234.

15.   Lynch G, Bahr BA, and Vanderklish PW (1991) Induction and stabilization of long-term potentiation. In Ascher P, Choi D, Christen Y (eds): *Glutamate, Cell Death and Memory*. Heidelberg, FRG: Springer-Verlag, pp 45-60.

16.   Bahr BA, Sheppard A, Vanderklish PW, Bakus BL, Capaldi D, and Lynch G (1991) Antibodies to the $\alpha_v\beta_3$ integrin label a protein concentrated in brain synaptosomal membranes. *NeuroReport* **2**:321-324.

17.   Bahr BA, Vanderklish PW, Ha LT, Tin M, Kessler M, and Lynch G (1991) Spectrin breakdown products increase with age in telencephalon of mouse brain. *Neurosci Lett* **131**:237-240.

18.   Xiao P, Bahr BA, Staubli U, Vanderklish PW, and Lynch G (1991) Evidence that matrix recognition contributes to stabilization but not induction of LTP. *NeuroReport* **2**:461-464.

19.   Bahr BA and Lynch G (1992) Purification of an Arg-Gly-Asp selective matrix receptor from brain synaptic plasma membranes. *Biochem J* **281**:137-142.

20.   Bahr BA and Parsons SM (1992) Purification of the vesamicol receptor. *Biochemistry* **31**:5763-5769.

21.   Bahr BA, Clarkson ED, Rogers GA, Noremberg K, and Parsons SM (1992) A kinetic and allosteric model for the acetylcholine transporter-vesamicol receptor in synaptic vesicles. *Biochemistry* **31**:5752-5762.

22.   Bahr BA, Noremberg K, Rogers GA, Hicks BW, and Parsons SM (1992) Linkage of the acetylcholine transporter-vesamicol receptor to proteoglycan in synaptic vesicles. *Biochemistry* **31**:5778-5784.

23.   Bahr BA, Godshall AC, Hall RA, and Lynch G (1992) Mouse telencephalon exhibits an age-related decrease in glutamate (AMPA) receptors but no change in nerve terminal markers. *Brain Res* **589**:320-326.

24.   Vanderklish P, Neve RL, Bahr BA, Arai A, Hennegriff M, and Lynch G (1992) Suppression of a glutamate receptor subunit impairs long-term potentiation. *Synapse* (NY) **12**:333-337.

25. Bahr BA, Vodyanoy V, Hall RA, Suppiramaniam V, Kessler M, Sumikawa K, and Lynch G (1992) Functional reconstitution of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors from rat brain. *J Neurochem* **59**:1979-1982.

26. Clarkson ED, Bahr BA, and Parsons SM (1993) Classical noncholinergic neurotransmitters and the vesicular transport system for acetylcholine. *J Neurochem* **61**:22-28.

27. Parsons SM, Bahr BA, Rogers GA, Clarkson ED, Noremberg K, and Hicks BW (1993) Acetylcholine transporter-vesamicol receptor pharmacology and structure. *Prog Brain Res* **98**:175-181.

28. Vodyanoy V, Bahr BA, Suppiramaniam V, Hall RA, Baudry M, and Lynch G (1993) Single channel recordings of reconstituted AMPA receptors reveal low and high conductance states. *Neurosci Lett* **150**:80-84.

29. Neve RL, Ivins KJ, Neve KA, Bahr BA, Vanderklish PW, Arai A, and Lynch G (1993) The use of antisense intervention to decipher the role of the neuronal growth-associated protein GAP-43. *Neuroprotocols* **2**:39-49.

30. Bahr BA, Godshall AC, Murray BA, and Lynch G (1993) Age-related changes in neural cell adhesion molecule (NCAM) isoforms in the mouse telencephalon. *Brain Res* **628**:286-292.

31. Hall RA and Bahr BA (1994) AMPA receptor development in rat telencephalon: [^{3}H]AMPA binding and Western blot studies. *J Neurochem* **63**:1658-1665.

32. del Cerro S, Arai A, Kessler M, Bahr BA, Vanderklish P, Rivera S, and Lynch G (1994) Stimulation of NMDA receptors activates calpain in cultured hippocampal slices. *Neurosci Lett* **167**:149-152.

33. Bahr BA, Lam N, and Lynch G (1994) Changes in the concentrations of tau and other structural proteins in the brains of aged mice. *Neurosci Lett* **175**:49-52.

34. Bahr BA, Neve RL, Sharp J, Geller AI, and Lynch G (1994) Rapid and stable gene expression in hippocampal slice cultures from a defective HSV-1 vector. *Mol Brain Res* **26**:277-285.

35. Bahr BA, Abai B, Gall C, Vanderklish PW, Hoffman KB, and Lynch G (1994) Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. *Exp Neurol* **129**:81-94.

36. Bahr BA, Tiriveedhi S, Park GY, and Lynch G (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *J Pharmacol Exp Ther* **273**:902-908.

37. Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Singh Mutneja M, Gall C, and Hoffman KB (1995) Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. *Hippocampus* **5**:425-439.

38. Bednarski E, Vanderklish PW, Gall CM, Saido TC, Bahr BA, and Lynch G (1995) Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. *Brain Res* **694**:147-157.

39. Bahr BA (1995) Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. *J Neuroscience Res* **42**:294-305.

40. Bahr BA, Hoffman KB, Kessler M, Hennegriff M, Park GY, Yamamoto R S, Kawasaki BT, Vanderklish PW, Hall RA, and Lynch G (1996) Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. *Neuroscience* **74**:707-721.

41. Hall RA, Vodyanoy V, Quan A, Sinnarajah S, Suppiramaniam V, Kessler M, and Bahr BA (1996) Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors. *Neurosci Lett* **217**:179-183.

42. Bahr BA, Staubli U, Xiao P, Chun D, Zhan-Xin J, Esteban ET, and Lynch G (1997) Arg-Gly-Asp-Ser-selective adhesion and the stabilization of long-term potentiation: Pharmacological studies and the characterization of the candidate matrix receptor synaptegrin-1. *J Neuroscience* **17**:1320-1329.

43. Capaldi D, Rosario R, Esteban ET, and Bahr BA (1997) A 27-kDa matrix receptor from rat brain synaptosomes: Selective recognition of the Arg-Gly-Asp-Ser domain and unique resistance to calcium-dependent proteolysis. *Neuroscience Res* **28**:275-279.

44. Kawasaki BT, Hoffman KB, Yamamoto RS, and Bahr BA (1997) Variants of the receptor/channel clustering molecule gephyrin in brain: Distinct distribution patterns, developmental profiles, and proteolytic cleavage by calpain. *J Neuroscience Res* **49**:381-388.

45. Edelstein CL, Ling H, Gengaro PE, Nemenoff RA, Bahr BA, and Schrier RW (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Internatl* **52**:1271-1278.

46. Bahr BA, Hoffman KB, Yang AJ, Hess US, Glabe CG, and Lynch G (1998) Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. *J Comp Neurol* **397**:139-147.

47. Bahr BA and Vicente JS (1998) Age-related phosphorylation and fragmentation events influence the distribution profiles of distinct tau isoforms in mouse brain. *J Neuropathol Exp Neurol* **57**:111-121.

48. Hoffman KB, Larson J, Bahr BA, and Lynch G (1998) Activation of NMDA receptors stimulates extracellular proteolysis of cell adhesion molecules in hippocampus. *Brain Res* **811**:152-155.

49.   Blomgren K, Hallin U, Andersson A-L, Puka-Sundvall M, Bahr BA, McRae A, Saido TC, Kawashima S, and Hagberg H (1999) Calpastatin is up-regulated in response to hypoxia, and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. *J Biol Chem* **274**:14046-14052.

50.   Sabatini DM, Barrow RK, Blackshaw S, Burnett PE, Lai MM, Field ME, Bahr BA, Kirsch J, Betz H, and Snyder SH (1999) Interaction of RAFT1 with gephyrin required for rapamycin-sensitive signaling. *Science* **284**:1161-1164.

51.   Sinnarajah S, Suppiramaniam V, Kumar KP, Hall RA, Bahr BA, and Vodyanoy V (1999) Heparin modulates the single channel kinetics of reconstituted AMPA receptors from rat brain. *Synapse* (NY) **31**:203-209.

52.   Bahr BA, Yilma S, and Suppiramaniam V (1999) Structural chemistries underlying synaptic signaling and regulation. *J Mol Biol Biotech* **1**:35-48 (http://www.jmbab.com/a5bahr.htm).

53.   Shi Y, Melnikov VY, Bahr BA, Schrier RW, and Edelstein CL (2000) Downregulation of the calpain inhibitor protein calpastatin by caspases during renal ischemia-reperfusion. *Am J Physiol Renal Physiol* **279**:F509-F517.

54.   Bahr BA (2000) Integrin-type signaling has a distinct influence on NMDA-induced cytoskeletal disassembly. *J Neuroscience Res* **59**:827-832.

55.   Rohn TT, Ivins KJ, Bahr BA, Cotman CW, and Cribbs DH (2000) A monoclonal antibody to amyloid precursor protein induces neuronal apoptosis. *J Neurochem* **74**:2331-2342.

56.   Vanderklish PW and Bahr BA (2000) The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Internatl J Exp Pathol* **81**:323-339.

57.   Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, and Vodyanoy V (2001) Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. *Synapse* (NY) **40**:154-158.

58.   Hoffman KB, Murray BA, Lynch G, Munirathinam S, and Bahr BA (2001) Delayed and isoform-specific effect of NMDA exposure on neural cell adhesion molecules in hippocampus. *Neuroscience Res* **39**:167-173.

59.   Rohn TT, Head E, Su JH, Anderson AJ, Bahr BA, Cotman CW, and Cribbs DH (2001) Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *Amer J Pathol* **158**:189-198.

60.   Bahr BA (2001) Adhesion chemistries and synaptic regulation. *Neurochemistry News* **1**:78-81.

61.   Wang X, Karlsson J-O, Zhu C, Bahr BA, Hagberg H, and Blomgren K (2001) Caspase-3 activation after neonatal rat cerebral hypoxia-ischemia. *Biol Neonate* **79**:172-179.

62. Blomgren K, Zhu C, Wang X, Karlsson J-O, Andersson A-L, Bahr BA, Mallard C, and Hagberg H (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia - A mechanism of "pathological apoptosis"? *J Biol Chem* **276**:10191-10198.

63. Caba E, Brown QB, Kawasaki B, and Bahr BA (2002) Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. *J Neuroscience Res* **67**:787-794.

64. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, and Rogers G (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp Neurol* **174**:37-47.

65. Bendiske J, Caba E, Brown QB, and Bahr BA (2002) Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J Neuropathol Exp Neurol* **61**:640-650.

66. Ferrand-Drake M, Karlsson J-O, Wieloch T, Bahr B, and Blomgren K (2002) Cyclosporin A prevents calpain and caspase-3 activation following hypoglycemia, implying mitochondrial permeability transition for protease activation in brain injury. *J Neuroscience*, in press.

67. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem*, in press.

68. Vicente JS, Munirathinam S, and Bahr BA (2002) Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age. *J Mol Biol Biotech* **3**:1-9 (http://www.jmbab.com/vol3p1pdf.pdf).

69. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O (2002) Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *J Eur Neurosci*, in press.

70. Bendiske J, and Bahr BA (2002) Activation of the lysosomal system: A compensatory response against protein deposition and associated synaptic pathogenesis. *J Neuropathol Exp Neurol*, in press.

71. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of AMPA-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol*, in press.

ABSTRACTS

1. Bahr BA, Anderson D, Parsons SM, Kinetic mechanism of a new anticholinergic that inhibits acetylcholine transport by synaptic vesicles, *Pacific Slope Biochem Conf*, IX-49 (1984).

2. Parsons SM, Anderson DC, Bahr BA, Quion R, Kirtman B, Nilsson LM, Rogers GA, Optimal structure of AH5183 for anticholinergic function, *Pacific Slope Biochem Conf*, IX-50 (1984).

3. Parsons, S.M., Anderson, D.C., Bahr, B.A., Nilsson, L.M., and Rogers, G.A., A Structure-Activity Study of AH5183, a New Anticholinergic Which Blocks Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **10**, 275 (1984).

4. Parsons, S.M., Anderson, D.C., Bahr, B.A., and Rogers, G.A., A New Type of Intraterminal Receptor at the Cholinergic Synapse, *FASEB Abstr. Fed. Proc.*, **43**, 289 (1984).

5. Parsons, S.M., Bahr, B.A., and Anderson, D.C., Mechanistic Studies of Acetylcholine Storage and Drug Inhibition, *Soc. Neurosci. Abstr.*, **11**, 347 (1985).

6. Parsons SM, Anderson DC, and Bahr BA, Acetylcholine Storage, Toxin and Drug Action in *Torpedo* Synaptic Vesicles, *Tenth Meeting of the Internatl. Soc. Neurochem.*, **38**, 389 (1985).

7. Bahr BA and Parsons SM, Acetylcholine storage inhibition and binding behavior of the anticholinergic AH5183 in synaptic vesicles, *Pacific Slope Biochem Conf*, II-14 (1985).

8. Parsons, S.M., Gracz, L.M., Bahr, B.A., and Kornreich, W.D., The AH5183 Receptor in VP_1 and VP_2 Cholinergic Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **12**, 239 (1986).

9. Bahr, B.A. and Parsons, S.M., The Vesamicol (AH5183) Receptor in VP_1 Cholinergic Synaptic Vesicles - Partial Purification, *Soc. Neurosci. Abstr.*, **13**, 670 (1987).

10. Bahr BA and Parsons SM, An acetylcholine binding site copurifies with the vesamicol (AH5183) receptor from cholinergic synaptic vesicles, *Soc. Neurosci. Abstr.*, **14**, 682 (1988).

11. Bahr, B.A. and Parsons, S.M., The Purified Vesamicol (AH5183) Receptor from Cholinergic Synaptic Vesicles May Contain an Allosteric Acetylcholine Binding Site, *NATO/FEBS Internatl. Meeting:Receptors, Membrane Transport, and Signal Transduction*, P-4 (1988).

12. Parsons SM, Yamagata S, Rogers G, and Bahr BA, Newly Identified Proteins of Cholinergic Synaptic Vesicle, *12th Meeting of the Internatl. Soc. Neurochem.*, **42**, 441 (1989).

13. Bahr, B.A. and Parsons, S.M., Characterization of the Vesamicol (AH5183) Receptor in VP_1 Cholinergic Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **15**, 814 (1989).

14. Parsons SM, Rogers G, Bahr BA, Yamagata S, Kornreich W, Regulation of vesicular storage of acetylcholine, *Internatl. Soc. Neurochem. Presynaptic Function Symposium*, 203 (1989).

15. Bahr BA, Acetylcholine storage system and drug inhibition in synaptic vesicles, *Dissertation Abstr. Internatl.*, Sec. B, University Microfilms Internatl., **vol. 147**, 872-873 (1989).

16.   Bahr BA, Staubli U, Lynch G, Transmembrane linkages between the extracellular matrix and the cytoskeleton affecting long-term synaptic modulation, *Eighth Internatl. Meeting on Cellular Mechanisms of Memory Formation and Long-Term Potentiation*, **8**, 12 (1990).

17.   Bahr BA, Bakus B, Lynch G, Identification of synaptic antigens from rat brain that are related to Arg-Gly-Asp binding adhesion proteins, *UCI/USC Joint Neurosci. Symposium*, **1** (1990).

18.   Lynch, G., Wu, J., Bahr, B.A. and Sheppard, A.M., Aspects of Calpain Biochemistry and Neuronal Membrane Plasticity, *Soc. Neurosci. Abstr.*, **16**, 1012 (1990).

19.   Bahr, B.A., Sheppard, A., Vanderklish, P.W., Bakus, B., Capaldi, D.K., Kessler, M., Ha, L.T., Tin, M.T., and Lynch, G., Fibronectin Binding to Brain Synaptosomal Membrane May Involve Novel Matrix Receptors, *Soc. Neurosci. Abstr.*, **16**, 1011 (1990).

20.   Bahr BA, Xiao P, Staubli U, Vanderklish P, Bakus B, Capaldi D, Sheppard A, Lynch G, Transmembrane linkages between the extracellular matrix and the cytoskeleton affecting LTP, *Fourth Internatl. Conference on the Neurobiology of Learning and Memory*, **4**, 49 (1990).

21.   Lynch G, Bahr BA, and Vanderklish PW, Disassembling and Reassembling Synapses: Some Points of Contact Between Plasticity and Pathology, *Fondation IPSEN pour la Recherche Therapeutique Paris Meeting: Glutamate, Cell Death and Memory*, **1**, 121 (1990).

22.   Bahr, B.A. and Lynch, G., Brain Synaptosomal Membranes Contain Novel Matrix Receptors with Conserved Integrin Epitopes, *Mol. Biol. Cell* **111** (Suppl.), 401a (1990).

23.   Bahr BA, Staubli U, Ambros-Ingerson J, Larson J, Kessler M, Xiao P, Godshall AC, Hall RA, Lynch G (1991) Receptor changes and LTP: Mechanisms of expression and stabilization and implications for age-related disorders of brain function. *Du Pont Biotech Update* **6**:63.

24.   Capaldi, D.K., Bahr, B.A., and Lynch, G., Identification of a Synaptosomal Proteoglycan that has an Arg-Gly-Asp Binding Site for Extracellular Matrix Components, *UCI/USC Joint Neurosci. Symposium*, **2** (1991).

25.   Bahr, B.A., and Lynch, G., Purification of the Matrix Recognition Molecule *F55* from Synaptic Membranes, *UCI/USC Joint Neurosci. Symposium*, **2** (1991).

26.   Clarkson, E.D., Bahr, B.A., Rogers, G.A., and Parsons, S.M., Kinetic and Allosteric Model for the Acetylcholine Transporter-Vesamicol Receptor: A Very Low Specificity Transporter, *Soc. Neurosci. Abstr.*, **17**, 1550 (1991).

27.   Vanderklish P, Kessler M, Hall R, Bahr BA, Sumikawa K, Lynch G, The AMPA/Quisqualate Receptor is a Substrate for Calpain, *Soc. Neurosci. Abstr.*, **17**, 1536 (1991).

28.   Ha, L.T., Bahr, B.A., Vanderklish, P.W., Tin, M.T., Murray, B., and Lynch, G., Spectrin Breakdown Product and Changes in N-CAM Increase with Age in Specific Brain Areas, *Soc. Neurosci. Abstr.*, **17**, 53 (1991).

29. Bakus, B.L., Bahr, B.A., Esteban, E.T., Godshall, A.C., Black, J., and Lynch, G., Groups of Structural Components Co-localized in Brain Subcellular Fractions are Differentially Affected by a Calcium Activated Protease, *Soc. Neurosci. Abstr.*, **17**, 1153 (1991).

30. Bahr BA, Xiao P, Lynch G, Evidence that the matrix recognition molecule *F55* is present in synapses and contributes to the stabilization of LTP, *Soc. Neurosci. Abstr.*, **17**, 387 (1991).

31. Capaldi, D.K., Bahr, B.A., Lim, H., Noremberg, K., and Lynch, G., A Proteoglycan-like Heparin Binding Protein of High Molecular Weight Found Concentrated in Synaptosomal Membranes, *Soc. Neurosci. Abstr.*, **17**, 1154 (1991).

32. Hall, R., Bahr, B.A., Kessler, M., Sumikawa, K., and Lynch, G., Partial Purification and Specific Immunoreactivity of the AMPA-type Glutamate Receptor from Rat Brain, *Soc. Neurosci. Abstr.*, **17**, 74 (1991).

33. Xiao P, Bahr BA, Staubli U, Ambros-Ingerson J, Hall RA, Kessler M, Larson J, Capaldi D, Esteban ET, Lynch G, Selective changes of AMPA receptors in the mechanisms of LTP expression and stabilization, *5th Conf on Neurobiol of Learning and Memory*, **5**, 14 (1992).

34. Vodyanoy V, Bahr BA, Suppiramaniam V, Hall R, Hennegriff M, Hoffman K, Baudry M, Lynch G, Ligand-Gated Ion Channel Activity from Rat Brain AMPA Receptors Reconstituted in Lipid Bilayers, *5th Conf on Neurobiol of Learning and Memory*, **5**, 34 (1992).

35. Clarkson, E.D., Bahr, B.A., and Parsons, S.M., Classical Noncholinergic Neurotransmitters and the Vesicular Transport System for Acetylcholine, *Soc. Neurosci. Abstr.*, **18**, 94 (1992).

36. McQuade D, Bahr BA, Kessler M, Esteban E, Bakus B, Hall R, Vanderklish P, Shaw G, Sumikawa K, Lynch G, Antibodies Against the GluR-A AMPA Receptor Subunit Label 98 and 57 kDa Proteins Concentrated in PSDs, *Soc. Neurosci. Abstr.*, **18**, 88 (1992).

37. Hennegriff, M., Bahr, B.A., Hall, R., Guthrie, K.M., Gall, C.M., Yamamoto, R.S., and Lynch, G., Antibodies to the GluR-A, GluR-B/C, GluR-D, and NMDA-R1 Glutamate Receptor Subunits: Western Blot Studies, *Soc. Neurosci. Abstr.*, **18**, 88 (1992).

38. Lam N, Bahr BA, Godshall A, Granger R, Lynch G, Different age-related decreases of ankyrin and spectrin occur in mouse telencephalon, *Soc. Neurosci. Abstr.*, **18**, 1487 (1992).

39. Vodyanoy, V., Bahr, B.A., Suppiramaniam, V., Hall, R.A., Kessler, M., Baudry, M., and Lynch, G., Rat Brain Glutamate (AMPA) Receptors Reconstituted in Lipid Bilayers Exhibit Low and High Conductance States, *Soc. Neurosci. Abstr.*, **18**, 652 (1992).

40. Bahr, B.A., Abai, B., Shahrestani, S., Viteri, S., and Lynch, G., Evidence that Brain Synaptic Membranes Contain a Receptor for the Alzheimer Amyloid βA4 Peptide, *Soc. Neurosci. Abstr.*, **18**, 1438 (1992).

41. Bahr BA and Lynch G, Involvement of Adhesion Molecules in the Functional Reorganization of Synaptic Contacts, *Fifth Winter Conference on Neural Plasticity*, **5**, 20 (1993).

42. Sharp, J., Bahr, B.A., Neve, R.L., Geller, A.I., and Lynch, G., The Use of Organotypic Hippocampal Cultures for the Study of HSV-mediated Gene Transfer, *The Third UCI/USC Joint Neurosci. Symposium*, **3** (1993).

43. del Cerro, S., Arai, A., Kessler, M., Bahr, B., Vanderklish, P., Rivera, S., and Lynch, G., Activation of Calpain by NMDA Receptor Stimulation in Organotypic Hippocampal Slice Culture, *Soc. Neurosci. Abstr.*, **19**, 1349 (1993).

44. Hall RA, Bahr BA, and Lynch G, Developmental Shift Toward Low-Affinity AMPA Binding Occurs During Synaptogenesis in Rat Telencephalon, *Soc. Neurosci. Abstr.*, **19**, 721 (1993).

45. Hoffman KD, Bahr BA, Rivera S, Vanderklish P, Hall RA, Kessler M, Arai A, Gall C, and Lynch G, Stable Maintenance of Glutamate Receptors and Other Synaptic Components in Organotypic Hippocampal Slice Cultures, *Soc. Neurosci. Abstr.*, **19**, 1357 (1993).

46. Bahr BA, Sharp J, Neve R, Geller A, Lynch G, A herpes simplex virus for gene transfer into neurons in organotypic hippocampal slice cultures, *Soc. Neurosci. Abstr.*, **19**, 1690 (1993).

47. Hennegriff, M., Hall, R.A., Bahr, B.A., Neve, R.L., and Lynch, G., Effects of Cyclothiazide on [^{3}H]AMPA Binding to AMPA Receptor Subunits Stably Expressed in HEK 293 Cells, *Soc. Neurosci. Abstr.*, **20**, 484 (1994).

48. Bahr, B.A., Abai, B., Gall, C., Vanderklish, P.W, Hoffman, K.B., Singh, M., and Lynch, G., Induction of β-Amyloid Containing Polypeptides and Concomitant Changes in Synaptic Properties in Rat Hippocampus, *Soc. Neurosci. Abstr.*, **20**, 48 (1994).

49. Bahr BA, Park GY, Singh M, and Lynch G, Induction of Aging and Excitotoxicity Processes in Long-Term Hippocampal Slice Cultures, *Mol. Biol. Cell*, **5** (Suppl.), 468a (1994).

50. Bahr, B.A., Xiao, P., Esteban, E.T., and Lynch, G., Adhesion Molecules and Hippocampal Plasticity: Integrin-Type Matrix Recognition Contributes to the Stabilization of LTP, Possibly Involving the RGDS-Selective Matrix Receptor *F55*, *Soc. Neurosci. Abstr.*, **21**, 1323 (1995).

51. Hoffman, K.B., Murray, B.A., and Bahr, B.A., Adhesion Molecules and Hippocampal Plasticity: Evidence that a Selective, Delayed Change in Neural Cell Adhesion Molecule-180 ($NCAM_{180}$) is Associated with LTP Mechanisms, *Soc. Neurosci. Abstr.*, **21**, 1323 (1995).

52. Bednarski, E., Vanderklish, P., Gall, C.M., Saido, T., Bahr, B.A., Shaw, G., and Lynch, G., Antisense Oligonucleotides to Calpain I mRNA Protect Cultured Hippocampal Slices from NMDA-Induced Pathophysiology, *Soc. Neurosci. Abstr.*, **21**, 608 (1995).

53. Suppiramaniam V, Sinnarajah S, Vodyanoy V, Hall R, Rogers G, Bahr BA, Modulation of Channel Open Time in Reconstituted AMPA Receptors, *Soc. Neurosci. Abstr.*, **21**, 81 (1995).

54. Suppiramaniam, V., Sinnarajah, S., Vodyanoy, V., Rogers, G., Lynch, G., and Bahr, B.A., The Benzoylpiperidine Compound BDP-5 Prolongs Single Channel Open Times of AMPA Receptors Reconstituted in Bilayers, *FASEB J (Suppl.) Exp. Biol. 95*, **9**, A373 (1995).

55. Sinnarajah, S., Suppiramaniam, V., Vodyanoy, V., Hall, R.A., and Bahr, B.A., Channel Modulating Effects of Heparin in AMPA Receptors Reconstituted in Bilayers, *FASEB J (Suppl.) Exp. Biol. 95*, **9**, A373 (1995).

56. Bahr BA, Hoffman K, Vanderklish P, Kawasaki B, Yang A, Hess H, Gall C, Glabe C, Lynch G, The Alzheimer's Aβ Peptide Induces CA1-Specific Aβ Immunostaining and Synaptic Decay in Hippocampal Slice Cultures, *Soc. Neurosci. Abstr.*, **22**, 1209 (1996).

57. Kawasaki BT and Bahr BA, Selective Calpain Inhibitors Reduce Residual Spectrin Proteolysis and the Delayed Loss of Pre- and Postsynaptic Markers When Applied After Severe NMDA Exposure in Cultured Hippocampal Slices, *Soc. Neurosci. Abstr.*, **22**, 1907 (1996).

58. Bahr BA and Kawasaki BT, Hippocampal Slice Cultures as an *In Vitro* Model of Toxicity: Sensitive Assessment of Pathogenic Agents and Development of Therapeutics for Neurodegenerative Disorders, *The Pharmacologist* (ASPET), **39**, 48 (1997).

59. Bahr, BA, Vanderklish, P, Bednarski, E, Lynch, G, Calpain Activation in Long-Term Hippocampal Slices: A Model of Neurotoxicity, *First International Symposium on Calpains: Their Role in Pathology and New Therapeutic Opportunities* (1997).

60. Bahr BA, Kawasaki B, Quach C, Rogers G, Attenuation of excitotoxic damage by nanomolar levels of Ampakines in long-term hippocampal slices, *Soc. Neurosci. Abstr.*, **23**, 2306 (1997).

61. Suppiramaniam V, Kawasaki B, Causey Z, Yilma Y, Sinnaraja S, Vodyanoy V, Bahr BA, AMPA receptors exhibit corresponding changes in binding and channel properties induced by nanomolar levels of sulfated polysaccharides, *Soc. Neurosci. Abstr.*, **23**, 1203 (1997).

62. Edelstein CL, Ling H, Gengaro PE, Wangsiripaisan A, Bahr BA, Nemenoff RA, and Schrier RW, Calpain mediated changes in spectrin and NaK-ATPase in rat renal proximal tubules (PT), *J. Amer. Soc. Nephrol.* **8**, 2729 (1997).

63. Bahr BA, Kawasaki BT, and Hoffman KB, Variants of the receptor/channel clustering molecule gephyrin in brain: Distinct distribution patterns are influenced by calpain-mediated cleavage events. *Sixth Conference on the Neurobiology of Learning & Memory*, **6**, 17 (1997).

64. Bahr BA, Glabe C, Lynch G, Hippocampal CA1 neurons are selectively targeted by Aβ1–42 resulting in accumulation of carboxyterminal APP fragments and loss of synaptophysin. *IBC 7th Annual Conf. on Alzheimer's Disease: Breakthroughs from Leaders in the Field* (1998).

65. Suppiramaniam V, Prem Kumar S, Yilma S, Sinnarajah S, Vodyanoy V, Manivannan K, Bahr B, Polysaccharides Enhance the Cooperative Gating Behavior of AMPA Receptor Channels Reconstituted in Lipid Bilayers, *Soc. Neurosci. Abstr.*, **24**, 98 (1998).

66. Bahr BA, Calpain-Mediated Cytoskeletal Disassembly Following Brief NMDA Exposure is Attenuated by an Integrin Antagonist in Hippocampus, *Soc. Neurosci. Abstr.*, **24**, 866 (1998).

67. Blomgren K, Hallin U, Puka-Sundvall M, Bahr BA, Hagberg H, Selective interactions between caspase-3, calpain, and stress protein calpastatin in neonatal rat hypoxia-ischemia, *XIXth Internatl. Conf. on Cerebral Blood Flow and Metabolism, Brain '99;* Denmark (1999).

68. Blomgren K, Hallin U, Puka-Sundvall M, Bahr BA, and Hagberg H, Caspase-Calpain Interaction and Calpastatin as a Stress Protein in Neonatal Rat Hypoxia-Ischimia, *XIXth International Symposium on Cerebral Blood Flow and Metabolism, Brain '99* (1999).

69. Bendiske J, Rogers G, Rudin M, Urwyler S, Sauter A, and Bahr BA, Ampakine Modulators of AMPA-Type Glutamate Receptors Promote Neuronal Recovery after Excitotoxic Injury in Brain Tissue, *Mol. Biol. Cell,* **10** (Suppl.), 230 (1999).

70. Suppiramaniam V, Yilma S, Bowens A, Manivannan K, Bahr BA, and Dityatev A, Colominic Acid (polysialic acid) Alters the Channel Properties of AMPA Receptors Reconsitituted in Lipid Bilayers, *Soc. Neurosci. Abstr.,* **25**, 1489 (1999).

71. Blomgren K, Wang X, Hagelberg C, Karlsson J-O, Bahr BA, and Hagberg H, Caspase-3-calpain interaction in neonatal rat hypoxia-ischemia, *Soc. Neurosci. Abstr.,* **25**, 1585 (1999).

72. Bahr BA, Bendiske J, Rudin M, Urwyler S, Sauter A, and Rogers G, Positive Modulation of AMPA Receptors Promotes Cellular Repair Following Excitotoxic Injury to Brain Tissue *In Vitro* and *In Vivo, The Toxicologist* (Society of Toxicology), **54**, 179 (2000).

73. Bendiske J, Caba E, and Bahr BA, Tau modifications and concomitant transport disturbances following lysosomal dysfunction, *J. Neurochem.,* **74** (Suppl.), S30C (2000).

74. Brown QB and Bahr BA, Phosphorylation of the ERK1/ERK2 MAP kinase can be influenced by AMPA-type glutamate receptors and their positive modulation in hippocampus, *J. Neurochem.,* **74** (Suppl.), S20D (2000).

75. Bahr BA, Bendiske J, Brown QB, Rogers G, Rudin M, Urwyler S, and Sauter A, Multifaceted properties of AMPA receptors include links to neuroprotective signaling pathways, *J. Neurochem.,* **74** (Suppl.), S50C (2000).

76. Caba E, Brown QB, and Bahr BA, Evaluation of mitogen-activated protein kinase and NF-κB in an *in vitro* model of neuroprotection, *J. Neurochem.,* **74** (Suppl.), S20C (2000).

77. Bendiske J, Caba E, Lam S, Gianutsos G, Bahr BA, Tau deposition is linked to microtubule destabilization and concomitant synaptic decline, *Soc. Neurosci. Abstr.,* **26**, 1023 (2000).

78. Munirathinam S and Bahr BA, Positive modulation of hippocampal AMPA receptors promotes recovery several hours after trimethyltin-induced excitotoxicity, *Soc. Neurosci. Abstr.,* **26**, 503 (2000).

79. Bahr BA, Bendiske J, Brown QB, Caba E, Rogers G, Rudin M, Urwyler S, and Sauter A, Links to metabotropic signaling allow AMPA receptors to counteract the neurodegeneration resulting from their own ionotropic over-activity, *Soc. Neurosci. Abstr.,* **26**, 503 (2000).

80. Suppiramaniam V, Manivannan K, Subramaniam T, Dityatev A, and Bahr BA, Interaction between AMPA receptor channels: A mechanism for modifying synaptic strength, *Soc. Neurosci. Abstr.*, **26**, 902 (2000).

81. Blomgren K, Wang X-Y, Zhu C-L, Karlsson J-O, Bahr BA, Hagberg H, Activation of caspase-2, -3, -8, -9 in neonatal rat hypoxia-ischemia, *Soc. Neurosci. Abstr.*, **26**, 245 (2000).

82. Bahr BA, Bendiske J, Caba E, A link between microtubule and synaptic failure in a model of AD-type abnormal protein processing, *Proc. Australian Neurosci. Soc.*, **12**, 50 (2001).

83. Bahr BA and Munirathinam S, A Novel Protective Signaling Pathway Identified in a Model of Neurotoxin Exposure, *Dept. of Defense Workshop of the Neurotoxin Exposure Treatment Research Program (NETRP)* (2001).

84. Caba E, Giardina C, and Bahr BA, Evidence of a Biphasic NF-κB Response to an Excitotoxin in Hippocampus, *The Toxicologist* (Society of Toxicology), **55**, 1732 (2001).

85. Bahr BA and Munirathinam S, Multiple Avenues of Protection Identified in a Model of Neurotoxin Exposure, *The Toxicologist* (Society of Toxicology), **55**, 899 (2001).

86. Chicoine L, Gianutsos G, and Bahr BA, Sulfated glycosaminoglycan enhances the channel properties of calcium-permeable AMPA receptors, *New England Pharmacol Meeting* (2001).

87. Caba E and Bahr BA, Biphasic response of NF-κB to stroke-type pathogenesis in organotypic hippocampal slice cultures, *GRASP Pharmacy Research Conference* (2001).

88. Bahr BA, Structural Chemistries and Synaptic Plasticity, *J Neurochem* **78** (Suppl 1), 117 (2001).

89. Brown QB, Bendiske J, Caba E, Munirathinam S, and Bahr BA, AMPA-type glutamate receptors are linked to endogenous protection mechanisms resulting in reduced excitotoxic risk, *J Neurochem* **78** (Suppl) (2001).

90. Bendiske J and Bahr BA, Characterization and slowing of the protein deposition • transport failure • synaptic decay cascade in hippocampal slice cultures, *Second Neurobiology of Aging Conference: Identifying Accelerators and Brakes of Brain Aging* (2001).

91. Caba E, Giardina C, Rogers G, Bahr BA, Ampakine effects indicate that the biphasic NF-κB response to excitotoxicity denotes two opposing signaling pathways, *Soc. Neurosci. Abstr.*, **27**, 867.2 (2001).

92. Suppiramaniam V, Subramaniam T, Kanju PM, Kleene R, Moss A, Premkumar L, Brown Q, Bahr BA, and Dityatev A, Single channel recordings from mossy fiber synaptosomes: Evidence for the presence of high-affinity synaptic AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 502.10 (2001).

93. Subramaniam T, Kleene R, Manivannan K, Dityatev A, Bahr BA, and Suppiramaniam V, Polysialic acid modulates single channel properties of synaptosomal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 610.5 (2001).

94. Chicoine L, Suppiramaniam V, Gianutsos G, Kawasaki BT, and Bahr BA, Sulfated polysaccharides modulate calcium permeability and binding properties of hippocampal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 480.6 (2001).

95. Bahr BA, Bendiske J, Michaelis ML, Georg G, Caba E, and Brown Q, Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders, *Soc. Neurosci. Abstr.*, **27**, 581.2 (2001).

96. Bendiske J and Bahr BA, Positive lysosomal modulation reverses the PHF-tau accumulation • microtubule destabilization • transport failure • synaptic deterioration cascade, *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001).

97. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, The majority of cell death occurs early after intracerebral grafting of ventral mesencephalic tissue: A role for calpain activation. *Soc. Neurosci. Abstr.*, **27**, 197.4 (2001).

98. Bahr BA, Caba E, Giardina C, Brown QB, and Bendiske J, Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways, *Mol. Biol. Cell*, **12** (Suppl.), 2233 (2001).

99. Munirathinam S and Bahr BA, Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *The Toxicologist* (Society of Toxicology), **58**, 1551 (2002).

Survival Signaling and Selective Neuroprotection Through Glutamatergic Transmission

Ben A. Bahr,*† Jennifer Bendiske,* Queenie B. Brown,* Subramani Munirathinam,* Ebru Caba,*
Markus Rudin,‡ Stephan Urwyler,‡ André Sauter,‡ and Gary Rogers§

*Department of Pharmaceutical Sciences and the Neurosciences Program, †Center for Drug Discovery, University of Connecticut,
Storrs, Connecticut; ‡Novartis Pharma AG, Basel, Switzerland; §Cortex Pharmaceuticals, Irvine, California*

In the brain, α-amino-3-hydroxy-5-methyl-4-isox-azolepropionate (AMPA) receptors mediate glutamatergic neurotransmission and, when intensely activated, can induce excitotoxic cell death. In addition to their ionotropic properties, however, AMPA receptors have been functionally coupled to a variety of signal transduction events involving Src-family kinases, G-proteins, and the mitogen-activated protein kinase (MAPK). In the present study, we tested whether AMPA receptors are linked to appropriate signaling events in order to prevent neuronal injury and/or enhance recovery. AMPA stimulation in hippocampal slice cultures caused the selective activation of MAPK through the upstream activator MAPK kinase (MEK). Inhibition of either component of the AMPA receptor–MAPK pathway potentiated cellular damage due to serum deprivation, suggesting that this pathway facilitates compensatory signals in response to injury. Correspondingly, positive modulation of AMPA receptors with the Ampakine 1-(quinoxalin-6-ylcarbonyl)piperidine (CX516) enhanced MAPK activation and reduced the extent of synaptic and neuronal degeneration resulting from excitotoxic episodes. CX516 was neuroprotective when infused into slices either before or after the insult. The Ampakine derivative also elicited neuroprotection in an *in vivo* model of excitotoxicity as evidenced by reduction in lesion size and preservation of two different types of neurons. Interestingly, the AMPA receptor–MAPK pathway selectively protects against excitotoxicity since enhancing the pathway did not protect against the nonexcitotoxic, slow pathology initiated by lysosomal dysfunction. The results indicate that glutamatergic communication is important for cellular maintenance and that AMPA receptors activate survival signals to counterpoise their own excitotoxic potential. © 2002 Elsevier Science (USA)

Key Words: AMPA receptors; Ampakine CX516; compensatory signals; excitotoxicity; MAP kinase; spectrin breakdown.

INTRODUCTION

AMPA-type glutamate receptors have been studied for their role in synaptic plasticity, learning and memory, and several types of neuropathologies. Glutamate is the major excitatory amino acid in the brain and this neurotransmitter facilitates the fast component of excitatory communication through AMPA receptors (19). AMPA receptors not only express ionotropic properties but also transmit extracellular signals to the nucleus of cultured neurons through the MAPK signaling pathway (18, 28, 37, 38). The connection between AMPA receptors and MAPK expands the potential roles for glutamatergic responses. The MAPK cascade is involved in a myriad of processes including hippocampal-mediated learning (4, 12) and the neuroprotective actions of nerve growth factor (21), estrogen (11, 34), and other hormones (13, 17, 21). While AMPA receptors provide the necessary hippocampal activity for memory encoding and retrieval (29), it is not known whether such glutamatergic transmission participates in neuroprotective survival signaling.

While excessive glutamatergic activity through AMPA receptors is no doubt a critical feature of the excitotoxic damage associated with ischemia (14, 27, 33), low-level stimulation of AMPA receptors by endogenous glutamate has been shown to enhance the survival of cultured hippocampal neurons (10). In addition, McKinney *et al.* (26) recently found evidence that AMPA receptor activation by spontaneous vesicular release of glutamate promotes synaptic maintenance in hippocampus. Because AMPA stimulation elevates trophic signaling through a MAPK-dependent pathway (18), we expected that AMPA receptors might activate repair mechanisms following pathogenic insults. Here, we demonstrate that AMPA receptors can be positively modulated to promote the MAPK signaling pathway and, correspondingly, to prevent fatal levels of cellular damage after pathogenic insults in hippocampal slice cultures and in the adult brain.

MATERIALS AND METHODS

Organotypic hippocampal slice cultures. Sprague–Dawley rat litters (Charles River Laboratories, Wilmington, Massachusetts) were housed in accordance with guidelines from the National Institutes of Health. Animals 11–12 days postnatal were sacrificed by isoflurane anesthesia and decapitation. Transverse slices of hippocampus (400 μm) were then quickly prepared, maintained on Millicell-CM inserts (Millipore Corporation, Bedford, MA), and periodically supplied with fresh media composed of 50% basal medium Eagle, 25% Earle's balanced salts, 25% horse serum, and defined supplements (6, 8). Slices were allowed to mature for 15–20 days in culture before used in experiments.

MAPK activation. Slice cultures were treated with or without 100 μM AMPA for 0.5–3 min in the absence or presence of 30 μM CNQX or 50 μM AP5. Other slices received 100 μM 1-(quinoxalin-6-ylcarbonyl)piperidine (CX516) for 5–60 min in the absence or presence of 50 μM PD 98059. The culture inserts were then rapidly flooded with ice-cold homogenization buffer containing 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 μM okadaic acid, 10 nM calyculin A, and the protease inhibitors aprotinin, leupeptin, bestatin, E-64, pepstatin A (each at 2 μg/mL), and 4-(2-aminoethyl)benzenesulfonyl fluoride (0.3 mM). Each condition used 8–10 groups of six to eight slices each. MAPK activation was assessed with antibodies that recognized the phosphorylated catalytic core of active ERK isoforms.

Induction of neuropathology. To test for potentiated neurodegeneration, cultured slices were treated with CNQX (30 μM) or the MEK inhibitor U0126 (20 μM) under control or serum-free conditions for 24 h. For induction of excitotoxic pathology, slices were preincubated for 2 h with or without CX516, at a concentration of 100 μM unless stated otherwise, and then subjected to a 15-min AMPA (100 μM) or 5-h TMT (100 μM) exposure. After removal of the toxins, the cultures were subsequently quenched with media containing 40 μM CNQX and 20 μM MK-801 for 20 min. Fresh media with or without CX516 was then supplied and the slices maintained in culture for 1–10 days before being prepared for immunoblotting and immunocytochemistry. To induce lysosomal dysfunction, 60 μM chloroquine was infused into slice cultures that were preincubated with or without 100 μM CX516. The chloroquine treatment continued for 3–9 days in the absence or presence of CX516. Treatment groups ($n = 4$–6 per time point) were staggered so that all slices were harvested on the same day, at which time they were washed thoroughly in serum-free media and prepared for subsequent analyses. All compounds were freshly solubilized and the solutions were titrated to pH 7.3 and sterile filtered.

Immunoblot analysis. Slice cultures were gently removed with a soft brush and homogenized in groups of six to eight using ice-cold homogenization buffer. Protein content was determined with a BSA standard and equal aliquots of the slice samples were denatured in sampling buffer for 5 min at 100°C, then separated by 4–16% SDS-PAGE and blotted to nitrocellulose. Immunodetection was accomplished by incubation overnight at 4°C with anti-active MAPK (Cell Signaling, Beverly, MA), monoclonal anti-ERK1/ERK2 (Zymed Laboratories, South San Francisco, CA), anti-active JNK2 (Promega, Madison, WI), affinity-purified antibodies to calpain-mediated spectrin fragment BDP$_N$ (9), monoclonal anti-synaptophysin (Boehringer Mannheim, Indianapolis, IN), and affinity-purified antibodies to the AMPA receptor subunit GluR1 (7). Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and color development used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Color development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Bands were scanned at high resolution to determine integrated density with BIOQUANT software (R & M Biometrics, Nashville, TN).

Histology. Slices were rinsed with 0.1 M phosphate buffer (PB), pH 7.4, and fixed for 2 h in cold PB plus 4% paraformaldehyde. Slices were then cryoprotected and carefully removed from the insert, and serial sections were cut at 20-μm thickness and mounted on gelatin-coated slides. Sections were immunolabeled with anti-active MAPK and anti-BDP$_N$ using the avidin–biotin-peroxidase technique (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as the chromogen (6, 8). To measure area of BDP$_N$ immunostaining (μm^2), threshold digitization was used utilizing the BIOQUANT image analysis system linked to an Olympus AX70 microscope. Thresholding was set so that >90% of the punctate immunoreactivity in the neuropil registered as area stained in AMPA-treated slices. The percentage of total immunostaining above threshold was similar in slices treated with or without CX516. The established threshold criteria consistently found no staining in control slices. For cell counts, sections from slices were stained with cresyl violet. The average number of neurons across three viewfields of 6.5×10^4 μm^2 per slice was obtained using the BIOQUANT system, and percentage loss was calculated by comparing treated vs untreated slice groups. In CA1, adjacent viewfields were selected in CA1b and the initial area of CA1c. For CA3, three adjacent viewfields were selected from the zone immediately before the dentate gyrus envelope. Granule cells of the stratum granulosum were counted in three adjacent viewfields selected from

the middle segment of the inner blade. Pyramidal neurons with characteristic morphological features, reduced cytoplasmic staining, and dense nuclear staining with inclusions were counted as pyknotic nuclei. Means ± SEM were determined from 8–16 slices per group and compared by t test or ANOVA.

NF-κB activation. Nuclei were prepared from cultured slices homogenized in ice-cold nuclear extract buffer consisting of 20 mM HEPES (pH 7.9), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.7% IGEPAL CA-630, 10% glycerol, 2 μg/mL leupeptin, 5 μg/mL aprotinin, and freshly added 0.1 mM PMSF and 0.5 mM DTT. Centrifugation at 6500 rpm was used to wash the pellet twice, and then the pellet material was resuspended and incubated in high-salt buffer containing 0.5 M KCl for 30 min at 4°C. After a final centrifugation at 13,000 rpm for 30 min, the supernatant was collected and protein concentration determined with a BSA standard. Equal protein (7.5 μg) was added to the electrophoretic mobility shift assay mixture containing 166 μg/mL poly(dIdC) and 50 μg/mL BSA. The mixture was incubated on ice for 20 min and then at room temperature for 15 min with the P^{32}-labeled consensus oligonucleotide recognized by NF-κB (Promega). Supershift analyses included incubating samples for 20 min with antibodies to the p50 subunit (Santa Cruz Biotechnology, Santa Cruz, CA) before DNA–protein complexes were resolved by 4% native PAGE and visualized by Kodak X-OMAT film autoradiography.

In vivo excitotoxicity. In adult male rats under pentobarbital anesthesia (8–10 per treatment), 50 nmol AMPA in 2 μL PBS were coinjected with 0–900 nmol CX516 slowly into the right striatum. Control animals received injections of vehicle only or 600 nmol CX516. Lesion extent (mean volume ± SEM) was determined 24 h postinjection by magnetic resonance imaging utilizing a Biospec 47/15 spectrometer (Bruker, Karlsruhe, Germany). The radiofrequency probe was an Alderman–Grant-type resonator with an inner diameter of 40 mm and 50 mm in length. Eight coronal sections of 1.5 mm thickness were analyzed between 2.5 and 13 mm anterior to the intraaural line. Image analysis was conducted in a blind manner and with the following parameters: 60-ms spin echo delay, 2-s repetition delay, and a spatial resolution of 0.16 × 0.16 mm^2 (viewfield of 40 × 40 mm^2). The lesion area in each section was measured by semiautomated segmentation based on intensity thresholding, and the lesion volume was calculated from the pixel dimension and total thickness of sections. At 7 days postinjection, the animals were sacrificed and the cortices and striata from ipsilateral and contralateral hemispheres were rapidly dissected, weighed, and prepared for enzymatic assays. Tissue homogenates were prepared in 0.5% Triton X-100, 0.1 mM pyridoxal phosphate, and 1 mM mercaptoethanol and assessed for choline acetyltrans-
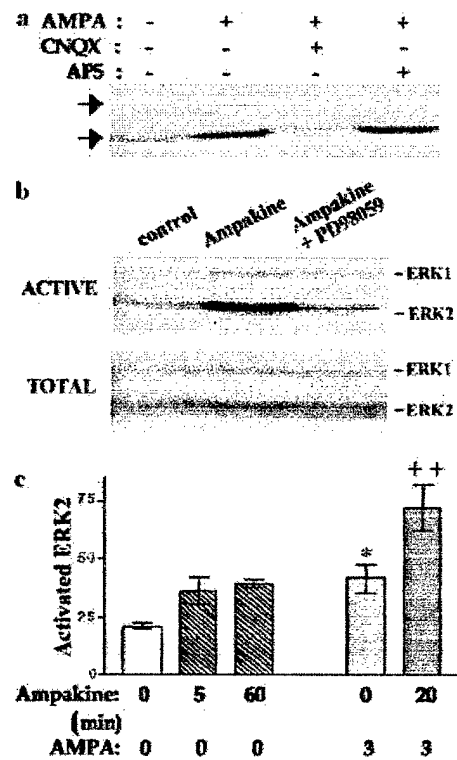

FIG. 1. AMPA receptors are linked to the activation of ERK1/ERK2 MAPK in hippocampal slice cultures. Untreated slices and slices exposed to AMPA for 2 min in the absence or presence of CNQX or AP5 were assessed for the phosphorylated active forms of ERK1 and ERK2. (b) Slices were treated with the AMPA receptor modulator CX516 for 60 min in the absence or presence of the inhibitor of MEK activation PD 98059. (c) Slices were treated with CX516 and AMPA for the noted times and mean integrated density of the phosphorylated active form of ERK2 was determined by immunoblot (±SEM). Unpaired, two-tailed t tests: $*P < 0.005$ compared to untreated slices; $++P = 0.01$ compared to AMPA-treated slices.

ferase (CAT) and glutamic acid decarboxylase (GAD) activities (16, 24). The percent change in enzymatic activity in the injected vs uninjected sides of the brains was determined (mean ± SEM).

Statistical analyses. Mean integrated densities for antigens in slice samples treated with or without an inhibitor were compared using unpaired, two-tailed t tests. Ampakine-induced immunostaining of phospho-ERK2, was compared to the integrated density from control samples using the unpaired, two-tailed t test. The difference between the mean area of BDP_N immunostaining in AMPA-insult slices vs control slices was determined by the two-tailed t test. Regional analyses of immunostained area or percentage cell loss were assessed by one-way ANOVA. Neuroprotective effects on the mean number of neurons or pyknotic nuclei per 6.5 × 10^4-μm^2 viewfield were determined by unpaired t tests. Dose-dependent studies assessed protection against an excitotoxic insult *in vitro* or *in vivo* across three dosages of Ampakine using the one-way ANOVA.
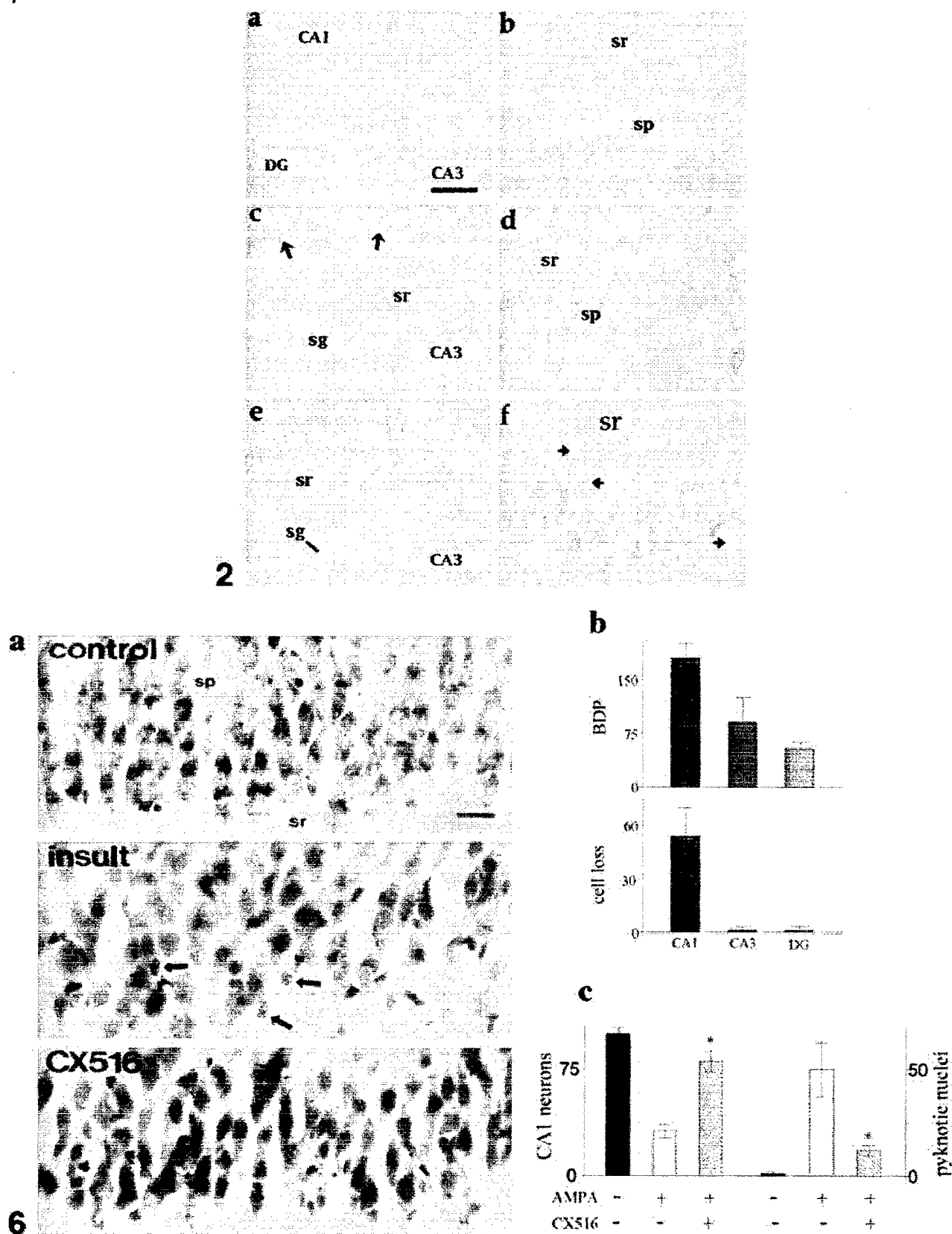
FIG. 2. (above) ERK1/ERK2 MAPK activation in hippocampal neurons. Control slices (a, b), slices treated with AMPA for 3 min (c) or with Ampakine CX516 for 5 min (d), and slices subjected to a 3-min AMPA exposure followed by 20 min with CX516 (e, f) were fixed, sectioned, and stained with anti-active MAPK antibodies. Subfield CA1 is shown at high power in (b), (d), and (f). DG, dentate gyrus; sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: a, c, e, 500 μm; b, d, f, 50 μm.
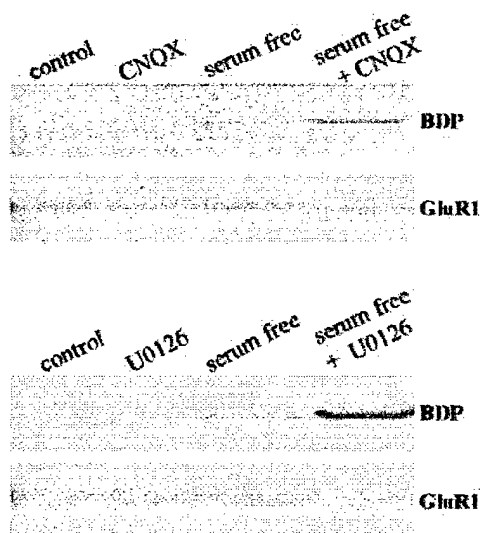
FIG. 3. Inhibition of the AMPA receptor-MAPK pathway promotes neurodegenerative processes. Cultured hippocampal slices were treated with the AMPA receptor antagonist CNQX or with the MEK inhibitor U0126 under control or serum-free conditions for 24 h, and then analyzed by immunoblot for calpain-mediated spectrin breakdown product (BDP) and the AMPA receptor subunit GluR1.

RESULTS

MAPK isoforms (ERK1/2) are activated when phosphorylated on Thr and Tyr residues of their catalytic core by the kinase MEK. Stimulation of AMPA receptors in hippocampal slice cultures caused rapid MAPK activation as indicated by enhanced staining of ERK1 (p44) and ERK2 (p42) with an antibody that specifically recognizes the dually phosphorylated isoforms (Fig. 1a). Increased MAPK activation found after as little as 30 s was similar to that measured after 2–3 min of AMPA exposure, and the effect was blocked by the AMPA receptor antagonist CNQX ($P = 0.01$) but not by the N-methyl-D-aspartate (NMDA) receptor antagonist AP5. These results indicate that stimulation of AMPA receptors modulates the MAPK signaling pathway in hippocampus.

AMPA receptor activation can be positively influenced by allosteric modulators that increase glutamate-mediated neurotransmission (2, 3, 35). Such positive modulation by the Ampakine CX516 also increased the levels of active ERK1/ERK2 (Fig. 1b) and

this induction was completely blocked by PD 98059 ($-122 \pm 11\%$, mean $\pm$ SEM; $P < 0.0001$), a specific inhibitor of MEK activation (1). Immunodetection of total MAPK (active and inactive ERK1/ERK2) in parallel immunoblot samples indicated that no change in MAPK protein levels was produced by AMPA (data not shown) or CX516 alone (Fig. 1b) during the time of kinase activation. In addition, the activation of MAPK was not associated with modulation of the c-Jun N-terminal protein kinase (JNK) pathway. AMPA treatment in cultured hippocampal slices had no effect on the level of phosphorylated active JNK2 enzyme ($98 \pm 17\%$ of levels in control slices), nor were the levels changed by positive modulation of AMPA receptors ($104 \pm 19\%$).

Next, we tested whether allosteric modulation promotes AMPA-induced activation of MAPK. Slice cultures treated with CX516 for 5–60 min exhibited an approximate twofold increase in activated ERK2 levels ($P < 0.0001$; Fig. 1c). The Ampakine treatment was able to sustain the enhanced phosphorylation of MAPK isoforms for several hours (data not shown), at a level similar to the twofold increase produced by brief AMPA exposure (Fig. 1c). Interestingly, CX516 further enhanced the AMPA-induced MAPK activation by 75%, thereby causing a total increase in activated ERK2 of 3.5-fold over the control levels ($P < 0.0005$; Fig. 1c). While only faint anti-active MAPK immunostaining was observed in control slices (Figs. 2a and 2b), phospho-MAPK-immunopositive cells were abundant in the stratum pyramidale of field CA1 in slices exposed to AMPA for 3 min (noted by arrows in Fig. 2c). The immunostaining was less intense in the CA3 subfield and the dentate gyrus, and no specific immunoreactivity was detected when the primary antibody was eliminated from the staining procedure. Phospho-MAPK staining in slices incubated with the Ampakine CX516 alone exhibited similar regional selectivity as that produced by AMPA, and the immunoreactivity was primarily restricted to the cytoplasm of pyramidal neurons (Fig. 2d). The additive effect of CX516 and AMPA was pronounced in field CA1 (Fig. 2e), where the phospho-MAPK immunostaining appeared to be both somatodendritic and nuclear (Fig. 2f; arrows indicate labeled apical dendrites).

FIG. 6. (previous page) Positive modulation of AMPA receptors by CX516 promotes cell survival. (a) Hippocampal slice cultures were treated with (insult) or without (control) AMPA for 15 min following the protocol described under Materials and Methods. A subset of the insulted group of slices received CX516 2 h prior to the insult. The slices were fixed 24 h postinsult and then sectioned and stained with cresyl violet. Photomicrographs of field CA1 are shown and pyknotic nuclei are noted with arrows (scale bar: 27 μm). (b) Calpain-mediated spectrin BDP was assessed as in Fig. 5, and cell counts were determined for Nissl-stained pyramidal neurons and granule cells in those slices subjected to the AMPA insult only. Specific BDP immunoreactivity was determined by image analysis and percent cell loss was calculated as compared to control slices (mean $\pm$ SEM). (c) Counts of pyramidal neurons (left three bars) and pyknotic nuclei (right bars) in area CA1 were determined to assess CX516-mediated neuroprotection. The mean number per 6.5×10^4-μm^2 viewfield is shown for each condition ($\pm$SEM). *$P < 0.01$, unpaired t test compared to insult-alone data.
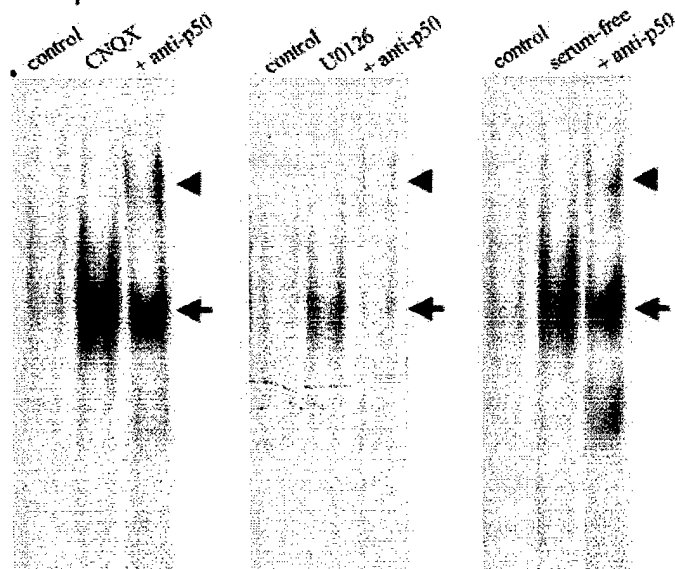
FIG. 4. Inhibition of the AMPA receptor–MAPK pathway promotes NF-κB activation. Cultured slices were subjected to the AMPA receptor antagonist CNQX, the MEK inhibitor U0126, or serum deprivation for 24 h. The slices were harvested and nuclear protein extracts were assessed for the DNA binding activity of NF-κB in the absence (arrows) or presence of anti-p50 antibodies as described under Materials and Methods. For each of the three conditions, the activated NF-κB contains the p50 subunit as supported by the supershift (arrowheads).

Cell-surface signal transduction through the link between AMPA receptors and MAPK may play a role in neuronal maintenance. In support of this, hippocampal slice cultures were found to be more vulnerable to neurodegeneration when AMPA receptors were blocked by CNQX for 24 h. Spectrin breakdown product BDP, a marker of excitotoxicity and other types of neurodegenerative events (36), was specifically labeled with antibodies to the cleavage site recognized by the calcium-dependent protease calpain (9). CNQX increased the level of spectrin BDP resulting from serum deprivation, and caused a corresponding ~50% decline in the carboxyl-terminal staining of postsynaptic marker GluR1 (Fig. 3, upper blots). The serum deprivation-induced damage may involve excitotoxic mechanisms as previously indicated in dopaminergic neurons (31). CNQX also caused activation of NF-κB (Fig. 4), a transcription factor known to be activated in response to pathogenic episodes (25). Similar evidence of enhanced vulnerability was found when the MAPK component of the AMPA receptor–MAPK pathway was disrupted with the MEK inhibitor U0126. Spectrin breakdown exhibited a pronounced increase and the GluR1 labeling decreased by 60% when serum deprivation was combined with U0126 for 24 h (Fig. 3, lower blots). MEK inhibition alone also caused NF-κB activation of similar magnitude as that produced by the withdrawal of serum (Fig. 4). These data indicate that

the AMPA receptor–MAPK link is important for cellular maintenance.

Since disruption of the AMPA receptor–MAPK pathway potentiated neurodegeneration, we tested whether enhancing this pathway promotes neuroprotective compensatory signals. For this purpose, we used the Ampakine modulator CX516 to enhance the AMPA receptor–MAPK pathway in the cultured hippocampal slice preparation. Slice cultures were exposed to the agonist AMPA for 15 min, and then the intense neuronal stimulation was rapidly quenched with glutamate receptor antagonists before slices were monitored for cellular damage. Immunocytochemical analysis with antibodies to calpain-mediated spectrin BDP revealed that CA1 neurons and dendritic puncta exhibit marked levels of cytoskeletal proteolysis 24–36 h after the excitotoxic insult (Fig. 5, middle). A separate group of slices was preincubated with the allosteric modulator CX516 and continually exposed to the Ampakine during the AMPA treatment and postinsult period. As shown in the bottom panels of Fig. 5, CX516 caused a dramatic decrease in the number of neurons exhibiting cytoskeletal damage after the recovery period. Total BDP immunostaining was significantly reduced in both the stratum pyramidale layer ($P < 0.01$) and the synapse-rich dendritic zone of the stratum radiatum ($P < 0.03$) as determined by image analysis.

Cytoskeletal degradation resulting from the excitotoxic exposure was associated with cell death. Cresyl violet-stained sections from hippocampal slice cultures revealed a decrease in CA1 neuron density as well as an increase in pyknotic nuclei 24 h postinsult (Fig. 6a). Upon examining the three hippocampal subfields (Fig. 6b), CA1-selective damage was evident with regards to both BDP immunostaining (ANOVA: $P = 0.01$) and percent cell loss ($P = 0.001$). Thus, a correspondence exists between the vulnerability to spectrin breakdown and cell death, suggesting that once a certain threshold of cytoskeletal damage is surpassed, the likelihood of cellular recovery is reduced. Related to this, the ability of the Ampakine modulator CX516 to reduce the extent of cytoskeletal breakdown after an excitotoxic insult indeed corresponds with its ability to prevent neuronal death and pyknotic changes (Figs. 6a and 6c).

The neuroprotective action of Ampakine CX516 was tested for selectivity in hippocampal slice cultures. As in the case against the excitotoxic action of AMPA, CX516 promoted recovery from exposure to TMT, a potent neurotoxin that initiates seizures and selective damage to hippocampal field CA3 by the overstimulation of glutamate receptors (9, 15, 20). Slice samples were prepared 24 h postinsult and analyzed for BDP, synaptophysin, and the AMPA receptor subunit GluR1 (Fig. 7a). CX516 almost completely eliminated the spectrin breakdown and the reductions in pre- and postsynaptic markers produced by a 5-h TMT exposure. The neuroprotectant action was dose-dependent
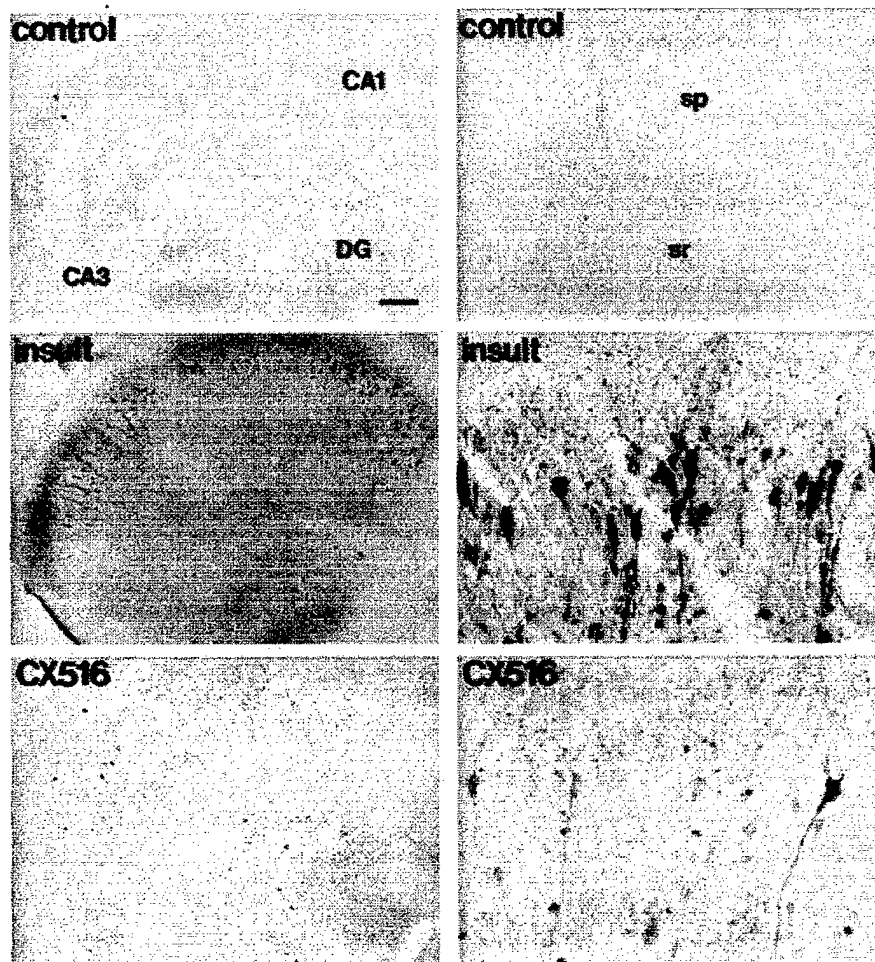
FIG. 5. Positive modulation of AMPA receptors by CX516 promotes cytoskeletal repair. Cultured slices were untreated (control) or subjected to a 15-min exposure to AMPA (insult) as detailed under Materials and Methods. The third group of slices had CX516 present throughout the experiment beginning 2 h prior to the insult. Slices were analyzed for calpain-mediated spectrin breakdown product 36 h postinsult. Field CA1 is shown at high power on the right. DG, dentate gyrus; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: left, 300 μm; right, 40 μm.

and similar in extent to that produced against AMPA-induced excitotoxicity (Fig. 7b). Cytoskeletal protection was evident against both types of toxic exposures (ANOVA: $P < 0.0001$ each) when comparing blot samples from insulted slices ($n = 14$–27 samples) with those that also received CX516 ($n = 10$–17). Ampakine modulation also enhanced synaptic maintenance in a dose-dependent manner in the TMT-insult ($P < 0.0001$) and AMPA-insult slices ($P = 0.01$), resulting in synaptic marker levels similar to those found in control slice cultures ($n = 18$–22). These results are of particular significance because CX516 was not infused into the slices until *after* the 5-h insult in the TMT experiment, whereas it was preincubated with slices *before* the AMPA insult. When AMPA receptor modulation was initiated after the 15-min AMPA exposure, the resulting reduction in cytoskeletal breakdown (-70%; $P < 0.001$) and recovery of synaptophysin (91%; $P < 0.001$) were comparable to the protection elicited by the preincubation protocol.

The above data indicate that modulation of AMPA receptors and the AMPA receptor–MAPK pathway elicits protection against pathogenic events involving the excitotoxic activation of glutamate receptors. Surprisingly, the allosteric modulation had no protective effect against nonexcitotoxic pathology. That is, the gradual loss of synaptic markers that occurs with lysosomal perturbation (6) was not influenced by CX516. Slice cultures incubated with the lysosomal inhibitor chloroquine exhibited steady declines in synaptophysin and GluR1 labeling after 3–9 days of treatment, and the declines were not attenuated by a 1-day preincubation and regular application of fresh CX516 (Fig. 7c). Six groups of slices treated with CX516 alone for 9 days expressed normal levels of the synaptic markers. More extensive dose–response analyses will be required to fully address possible differences in CX516 protection in acute vs chronic insult models.

Further studies were conducted to test whether positive modulation of AMPA receptors elicits protection
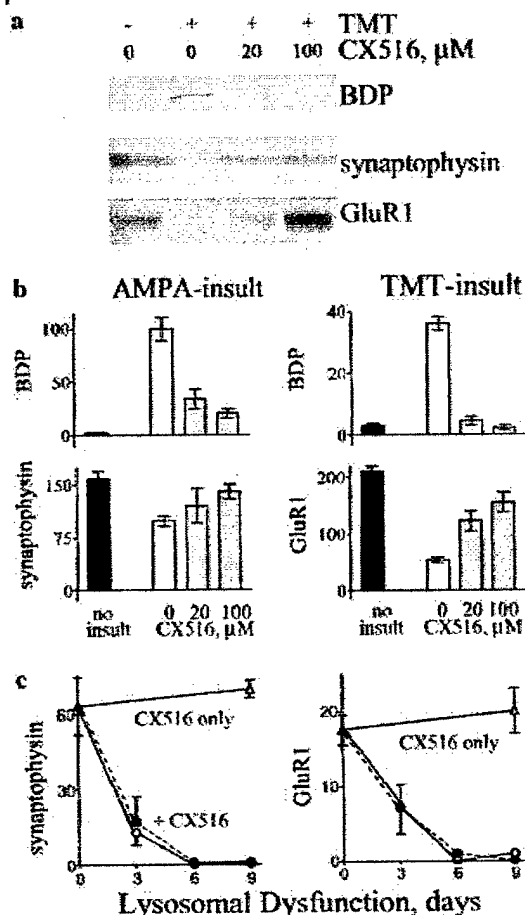
FIG. 7. Ampakine CX516 promotes selective neuroprotection in hippocampal slice cultures. (a) Slice cultures were treated with TMT for 5 h as described under Materials and Methods, and the 24-h recovery period was in the absence or presence of CX516. Slice samples were assessed by immunoblot for spectrin BDP, synaptophysin, and GluR1. (b) BDP and synaptic marker measures from the TMT-insult experiment are shown with those obtained from the 15-min AMPA-insult protocol where CX516 application was initiated before the insult (mean integrated densities ± SEM). (c) Slices were preincubated for 24 h with (closed circles) or without CX516 (open circles), followed by the addition of chloroquine to induce lysosomal dysfunction. Groups of slices were harvested and analyzed for synaptophysin and GluR1 levels (mean ± SEM).

against excitotoxicity *in vivo*. In order to initiate an excitotoxic insult in adult rats, 50 nmol of AMPA were locally applied to the right striatum with an injection track through the cortex. MRI analyses conducted 24 h later showed that the induced excitotoxicity produced marked unilateral damage (see arrow in Fig. 8b), while no lesion was evident in animals injected with vehicle only (Fig. 8a). Besides the expected damage in the striatum, administered AMPA extending into the injection track caused additional lesion formation in the cortex. When the Ampakine CX516 was coinjected with the excitotoxin, the lesion extent was dramatically reduced (Fig. 8c). The neuroprotection was found to be significant and dose-dependent (analysis of variance:

$P < 0.02$), attenuating the AMPA-induced lesion size by as much as 64% (Fig. 8e). As a control, CX516 injected alone caused no damage, in contrast to the sizable lesion volume produced by AMPA (see Fig. 8d). To assess long-term neuroprotection, ipsilateral and contralateral brain areas were dissected 7 days postinjection and enzyme markers for cholinergic (CAT) and GABAergic neurons (GAD) were measured. Within-animal comparisons found that the neuronal markers were significantly reduced in the lesioned vs unlesioned hemispheric tissue by 46–72% ($P < 0.01$). Corresponding with the MRI data, the enzyme reductions occurred in both the striatum and the adjacent cortical tissue. As shown in Fig. 8f, the decreases in CAT and GAD activities were completely attenuated by CX516 in the striatum (ANOVA: $P < 0.0001$ for both) and were significantly reduced in the neighboring cortical region as well ($P < 0.001$ and $P < 0.005$, respectively). Thus, Ampakine modulation elicits lasting protection against an excitotoxic insult.

DISCUSSION

The data presented indicate that glutamatergic activity through AMPA receptors is important for cell survival. Inhibition of AMPA receptors reduces cell growth (30) and causes an increased rate of neurodegeneration in cultured neurons (10), results consistent with those reported here regarding proteolytic responses and NF-κB activation in organotypic hippocampal slice cultures. The slice cultures offer the advantage that they exhibit stable features of the adult brain and, more importantly, they respond to pathogenic insults in the same way as found *in vivo* (5). Thus, the present report suggests that the same synaptic signals that help determine the composition of the developing brain are involved in the maintenance of mature brain tissue.

Enhanced glutamatergic transmission by the infusion of glutamate or AMPA has been shown to increase neuronal survival (10), attenuate apoptosis (23), and to prevent synaptic deterioration produced by deafferentation (26). Our results with the allosteric modulator CX516 indicate that AMPA receptor activation, including the low levels produced by spontaneous glutamate release, can be intensified in such a way as to strengthen related survival processes. Spectrin breakdown measures were valuable in evaluating structural repair after an excitotoxic exposure and, as expected, synaptic maintenance and cell survival correlated with the cytoskeletal protection. However, the survival systems activated through the modulation of AMPA receptors are selective with respect to the neuroprotection they elicit. Ampakine CX516 was assessed across different types of pathologies and its protective action was found to reduce the damage resulting from CA1- and CA3-targeting insults which involve the over-
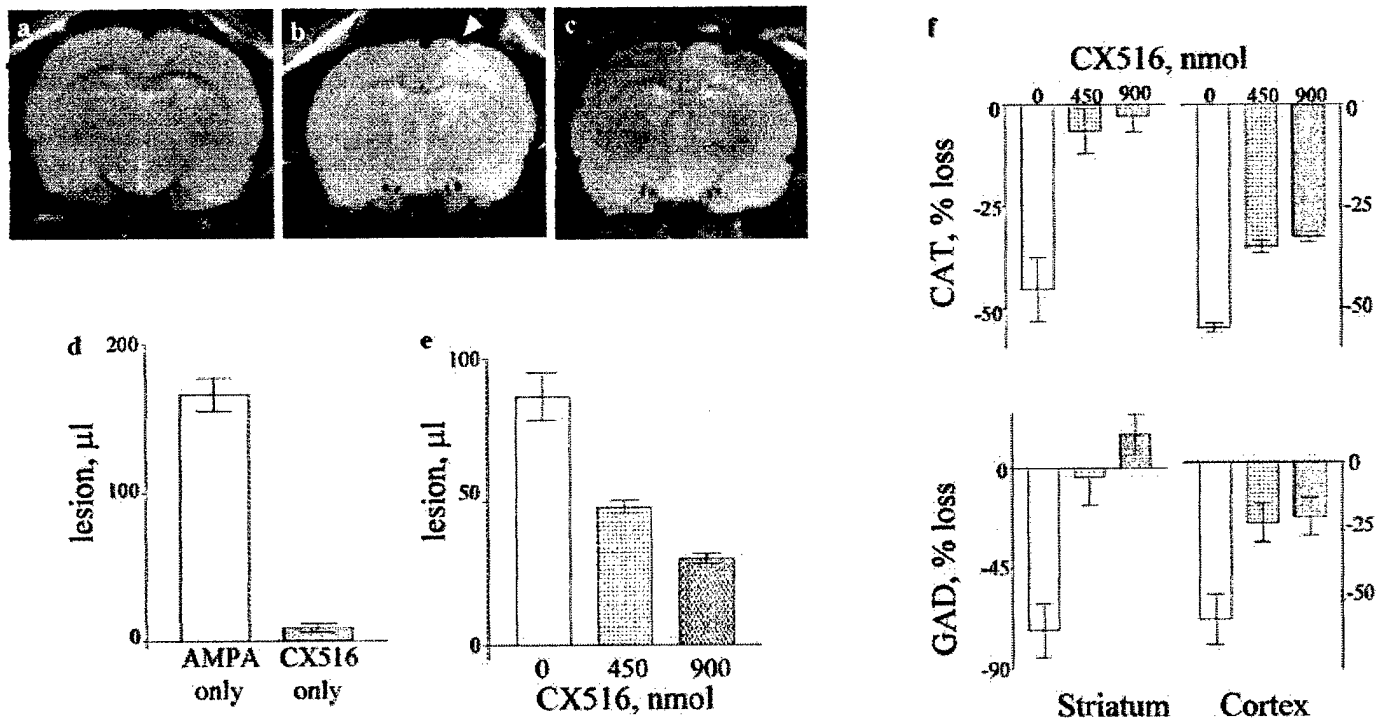
FIG. 8. Allosteric modulation of AMPA receptors elicits neuroprotection in adult rats. Animals were subjected to intrastriatal injections of CX516 (a), AMPA (b), or AMPA and CX516 together (c). Coronal sections were analyzed by MRI 24 h postinjection. (d) Lesion size was determined after injection of AMPA or CX516 alone (mean area ± SEM). (e) Dose-dependent reduction in AMPA-induced lesion extent was assessed in animals coinjected with CX516. (f) The activities of CAT and GAD were measured in dissected brain regions and expressed as the mean percent decrease in the lesioned side of the brain as compared to the unlesioned side (±SEM).

stimulation of glutamatergic receptors. In contrast, CX516 had no effect on another pathogenic mechanism, lysosomal dysfunction, which does not involve excitotoxic activity. The glutamate-mediated survival response, thus, can be exploited to prevent serious injury from a specific class of neuropathogenesis.

The signal transduction mechanism that translates AMPA receptor activity into survival signals specifically involves the ERK1/ERK2 MAPK. As shown, allosteric modulation promotes the AMPA receptors' ability to activate the MAPK signaling pathway in hippocampal neurons. While the AMPA receptor-MAPK pathway was evident in all hippocampal subfields, it was most apparent in field CA1. CX516-mediated modulation of the receptors after an excitotoxic insult protected those pyramidal neurons that exhibited enhanced levels of MAPK activation. MAPK's role in survival signaling was further supported by the fact that inhibition of either component of the AMPA receptor–MAPK pathway promotes cytoskeletal vulnerability and NF-κB responses.

The neuroprotection results using the cultured hippocampal slice suggest that the functional link between AMPA receptors and MAPK is important for injury-induced compensatory responses in the adult brain. In vivo studies also implicate glutamatergic and MAPK activity in neuronal protection. For example,

trisomy 16 mice, an animal model of Down syndrome, are reported to have a defect in a glutamate-mediated trophic response, resulting in accelerated cell death of hippocampal neurons (10). In addition, the MAPK pathway is necessary for the neuroprotection induced by sublethal preconditioning ischemia (32). As shown in the present study, Ampakine modulation of glutamatergic transmission protected the striatum and cortex from excitotoxic damage in the adult rat. In addition to the reduction in lesion extent, enzymatic markers measured 1 week after the insult indicated that the neuroprotection was not restricted to the neuronal type targeted by the Ampakine compound. Cholinergic and GABAergic neurons were completely protected in the striatum where the bulk of the Ampakine CX516 was coinjected with the excitotoxin. The extent of AMPA extending into the cortical injection track also caused sizable decreases in the neuronal markers, and CX516 attenuated these reductions. Expectedly, CX516 was higher in concentration in the striatum vs the cortex as indicated by the higher degree of neuroprotection elicited in the striatal area as compared to that found in cortical tissue. These findings suggest that glutamatergic communication between surviving neurons is vital for their full recovery and long-term maintenance after periods of excitotoxicity.

26. McKinney, R. A., M. Capogna, R. Durr, B. H. Gahwiler, and S. M. Thompson. 1999. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat. Neurosci.* **2:** 44–49.

27. Nellgard, B., and T. Wieloch. 1992. Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. *J. Cereb. Blood Flow. Metab.* **12:** 2–11.

28. Perkinton, M. S., T. S. Sihra, and R. J. Williams. 1999. Ca^{2+}-permeable AMPA receptors induce phosphorylation of cAMP response element-binding protein through a phosphatidylinositol 3-kinase-dependent stimulation of the mitogen-activated protein kinase signaling cascade in neurons. *J. Neurosci.* **19:** 5861–5874.

29. Riedel, G., J. Micheau, A. G. Lam, E. V. Roloff, S. J. Martin, H. Bridge, L. D. Hoz, B. Poeschel, J. McCulloch, and R. G. Morris. 1999. Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat. Neurosci.* **2:** 898–905.

30. Rzeski, W., L. Turski, and C. Ikonomidou. 2001. Glutamate antagonists limit tumor growth. *Proc. Natl. Acad. Sci. USA* **98:** 6372–6377.

31. Schierle, G. S., and P. Brundin. 1999. Excitotoxicity plays a role in the death of tyrosine hydroxylase-immunopositive nigral neurons cultured in serum-free medium. *Exp. Neurol.* **157:** 338–348.

32. Shamloo, M., A. Rytter, and T. Wieloch. 1999. Activation of the extracellular signal-regulated protein kinase cascade in the hippocampal CA1 region in a rat model of global cerebral ischemic preconditioning. *Neuroscience* **93:** 81–88.

33. Sheardown, M. J., P. D. Suzdak, and L. Nordholm. 1993. AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur. J. Pharmacol.* **236:** 347–353.

34. Singer, C. A., X. A. Figueroa-Masot, R. H. Batchelor, and D. M. Dorsa. 1999. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J. Neurosci.* **19:** 2455–2463.

35. Staubli, U., G. Rogers, and G. Lynch. 1994. Facilitation of glutamate receptors enhances memory. *Proc. Natl. Acad. Sci. USA* **91:** 777–781.

36. Vanderklish, P. W., and B. A. Bahr. 2000. The pathogenic activation of calpain: A marker and mediator or cellular toxicity and disease states. *Int. J. Exp. Pathol.* **81:** 323–339.

37. Wang, Y., and J. P. Durkin. 1995. α-Amino-3-hydroxy-5-methyl-4isoxazolepropionic acid, but not N-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein subunits in rat cortical neurons. *J. Biol. Chem.* **270:** 22783–22787.

38. Wang, Y., D. L. Small, D. B. Stanimirovic, P. Morley, and J. P. Durkin. 1997. AMPA receptor-mediated regulation of the G_i-protein in cortical neurons. *Nature* **389:** 502–504.

Peptidyl α-Keto Amide Inhibitor of Calpain Blocks Excitotoxic Damage Without Affecting Signal Transduction Events

Ebru Caba,[1] Queenie B. Brown,[1] Brian Kawasaki,[2] and Ben A. Bahr[1,2]★

[1]Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut
[2]Cortex Pharmaceuticals Inc., Irvine, California

The cysteine protease calpain is activated by calcium and has a wide range of substrates. Calpain-mediated cellular damage is associated with many neuropathologies, and calpain also plays a role in signal transduction events that are essential for cell maintenance, including the activation of important kinases and transcription factors. In the present study, the hippocampal slice culture was used as a model of excitotoxicity to test whether the neuroprotection elicited by selective calpain inhibition is associated with changes in cell signaling. Peptidyl α-keto amide and α-keto acid inhibitors reduced both calpain-mediated cytoskeletal damage and the concomitant synaptic deterioration resulting from an N-methyl-D-aspartate exposure. The α-keto amide CX295 was protective when infused into slice cultures before or after the excitotoxic episode. The slices protected with CX295 exhibited normal activation levels of mitogen-activated protein kinase and the transcription factor nuclear factor-κB. Thus, selective inhibition of calpain provides neuroprotection without influencing critical signaling pathways. © 2002 Wiley-Liss, Inc.

Key words: spectrin breakdown; calpain inhibition; NF-κB; MAPK; hippocampal slice culture

Calpains, a family of calcium-dependent cysteine proteases, play a functional role in cell cycle regulation, synaptic plasticity, and signal transduction (Saido et al., 1994; Santella et al., 1998; Chan and Mattson, 1999; Utz and Anderson, 2000). During signaling events, calpain has been shown to influence crucial elements, such as pp60^{src}, protein kinase C, phosphatase 1B, focal adhesion kinase, and several adhesion molecules (Saido et al., 1994; Cooray et al., 1996; Rock et al., 1997; Bahr et al., 1999). The protease is also linked to the transcription factor nuclear factor (NF)-κB, which is known to be activated in response to pathogenic episodes, perhaps as part of a compensatory response for promoting survival (see Mattson et al., 2000). Specifically, calpain has been implicated in the activation process of NF-κB (Shumway et al., 1999; Chen et al., 2000; Schoonbroodt et al., 2000). IκBα, an intrinsic inhibitor of NF-κB, is degraded by calpain, allowing for the nuclear translocation of the transcription factor as part of its activation. Unfortunately, calpain function is not restricted to signaling events underlying cellular maintenance but often triggers cascades leading to cellular damage.

Calpain activation plays a role in apoptosis (Villa et al., 1998; DeBiasi et al., 2001) and is a marker of a variety of pathogenic insults and disease states, including hypoxia/ischemia, epilepsy, stroke, and brain injury (for review see Vanderklish and Bahr, 2000). In many neuropathologies, calpain selectively targets the cytoskeletal protein spectrin, and the resulting breakdown product (BDP) can be measured as a sign of cellular damage using antibodies against spectrin's calpain recognition site (Saido et al., 1993; Roberts-Lewis et al., 1994; Bahr et al., 1995b). Calpain-mediated spectrin breakdown is a useful marker with which to assess cytoskeletal damage early after an excitotoxic insult, the marker increasing as pathophysiology and cell death occur and decreasing in correspondence with recovery or protection (Lee et al., 1991; Bednarski et al., 1995; Bahr et al., 2001; Neumar et al., 2001). Early neurodegeneration can also be monitored by measuring the concentration of synaptic proteins, such as synaptophysin and AMPA receptor subunits (Bahr et al., 1994, 1998; Bi et al., 1996, 1998), that exhibit significant alterations in accordance with the spectrin breakdown event (Bahr et al., 2001).

The involvement of calpain in neurodegeneration has prompted researchers to explore inhibitors of the protease in an attempt to elicit neuroprotection in response to

excitotoxicity. A variety of studies showed that nonselective cysteine protease inhibitors such as leupeptin and ALLN can block excitotoxic damage (Arai et al., 1990; Lee et al., 1991; Rami and Krieglstein, 1993; del Cerro et al., 1994; Bahr et al., 1995b; Posmantur et al., 1997). Alternatively, peptidyl α-keto amide, α-keto acid, and α-keto ester compounds were developed (Li et al., 1993, 1996) and demonstrated to block selectively the proteolytic responses of calpain. Selective calpain inhibitors are useful in that they provide protection for target neuronal populations (Bartus et al., 1994a, b; Hong et al., 1994; Brorson et al., 1995; Markgraf et al., 1998; Yokota et al., 1999). One compound in particular, the α-keto amide CX295, was shown to be especially effective in vivo, providing cellular and functional protection in models of ischemia and experimental brain injury (Bartus et al., 1994b; Saatman et al., 1996). By using the cultured hippocampal slice model of excitotoxicity (see Bahr, 1995), CX295 was evaluated for neuroprotective action as well as effects on relevant cell signaling pathways. With regard to the latter, activation of the ERK1/ERK2 mitogen-activated protein kinase (MAPK) and the transcription factor NF-κB were examined, because these two events represent rapid responses to excitotoxicity and also have been implicated in neuroprotection (Kotzbauer et al., 1996; Bonni et al., 1999; Han et al., 2000; Mattson et al., 2000).

MATERIALS AND METHODS

The hippocampus was dissected from 12-day-old Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described (Bahr et al., 1994, 1995b). Transverse slices of hippocampus (400 μm) were maintained in culture for 15–25 days on Biopore culture membranes (Millipore, Bedford, MA) in media composed of 50% basal medium Eagle, 25% Earle's balanced salt solution, 25% regular horse serum, and the following concentrations: 136 mM glutamine, 40 mM glucose, 0.5 mM ascorbic acid, 20 mM HEPES buffer (pH 7.3), 1 mg/liter insulin, 5 units/ml penicillin, and 5 mg/liter streptomycin. The medium was changed every 2 or 3 days, and the surface of the slices was exposed to humidified air plus 5% CO_2 at 37°C. Excitotoxicity was induced by infusing 200 μM N-methyl-D-aspartate (NMDA) into slices for 20 min. This concentration of NMDA was used in order to reproduce the type of rapid insult conducted routinely in dissociated neuronal cultures but using a slice of tissue with a thickness of 10–15 neurons (see Bahr et al., 1995a). The insult was followed by a rapid quenching step with glutamate receptor antagonists (40 μM MK801 and 40 μM CNQX), and slices were harvested or maintained in culture for 3 hr postinsult. The α-keto acid Z-Leu-Phe-COOH (CX270; 10 μM) and the α-keto amides Z-Leu-Abu-CONH-$(CH_2)_3$-Mpl (CX295; 10–100 μM) and Z-Leu-Nva-CONH-$(CH_2)_3$-Mpl (CX349; 100 μM) were synthesized as described elsewhere (Li et al., 1993). For pretreatment, the compounds were included in the slice media 30–60 min prior to the excitotoxic insult and remained present during the insult and postinsult. Posttreatment entailed the presence of calpain inhibitors only in the postinsult quenching and incubation steps. The cultured slices were harvested and homogenized

in ice-cold buffer composed of 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 10 μg/ml antipain, and freshly added phenylmethylsulfonyl fluoride (PMSF; 2 mM). Protein content was determined with a bovine serum albumin (BSA) standard curve. The level of spectrin breakdown in slice samples was assessed by immunoblot. Proteins were separated by 4–16% SDS-PAGE, transferred to nitrocellulose, and tested for breakdown product (BDP_N) using affinity-purified rabbit antibodies against end residues (Gln-Gln-Glu-Val-Tyr) of the amino-terminal spectrin fragment produced by calpain I (Bahr et al., 1995b). The postsynaptic marker GluR1 was labeled with antibodies made against its carboxy-terminal domain, Ser-His-Ser-Ser-Gly-Met-Pro-Leu-Gly-Ala-Thr-Gly-Leu, and affinity purified on immobilized peptide (Bahr et al., 1996). The presynaptic marker synaptophysin was immunolabeled with a mouse monoclonal antibody (Chemicon, Temecula, CA). MAPK activation was assessed using antibodies that recognized the phosphorylated catalytic core of active ERK isoforms (Cell Signaling, Beverly, MA). Blots were also stained for tubulin and with monoclonal anti-ERK1/ERK2 (Zymed Laboratories, South San Francisco, CA) to determine total ERK levels and ensure that equal protein was loaded. Integrated density of the bands was determined with Bioquant software (R & M Biometrics, Nashville, TN) after being scanned at high resolution, and the means ± SEM were compared using unpaired, two-tailed t-tests or ANOVA.

Anti-BDP_N immunocytochemistry was used to localize the spectrin breakdown fragments in sections from slice cultures as described elsewhere (Bahr, 2000). Slices were rinsed with 0.1 M phosphate buffer (PB), pH 7.4, and fixed for 2 hr in cold PB plus 4% paraformaldehyde. Slices were then cryoprotected and carefully removed from the insert, and serial sections were cut at 20 μm thickness and mounted on gelatin-coated slides. Sections were immunolabeled with anti-BDP_N using the avidin-biotin-peroxidase technique (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine as the chromogen.

Nuclear extractions were carried out as described by Schreiber et al. (1989), with modifications. Harvested slices were homogenized in ice-cold nuclear extract buffer [NEB; 20 mM HEPES (pH 7.9), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.7% IGEPAL CA630, 10% glycerol, 2 μg/ml leupeptin, and 5 μg/ml aprotinin, with freshly added 0.1 mM PMSF and 0.5 mM dithiothreitol (DTT)]. Homogenates were centrifuged at 6,500 rpm for 10 min, then resuspended and recentrifuged. The pellets were suspended in 35 μl ice-cold high-salt buffer (20 mM HEPES, pH 7.9, 0.5 M KCl, 0.5 mM EDTA, 25% glycerol, 2 μg/ml leupeptin, and 5 μg/ml aprotinin, with freshly added 0.5 mM DTT), vigorously shaken for 30 min at 4°C, and centrifuged at 13,000 rpm for 30 min. Supernatant was collected, and protein concentrations were determined. Equal amounts of protein were subjected to the electophoretic mobility shift assay as described by Kako et al. (1998). Briefly, 7.5 μg of nuclear proteins were incubated with 2.5 μg poly-dIdC and 0.75 μg BSA in a 15 μl total volume for 20 min, then incubated at room temperature for 15 min with ^{32}P-labeled NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega, Madison, WI).
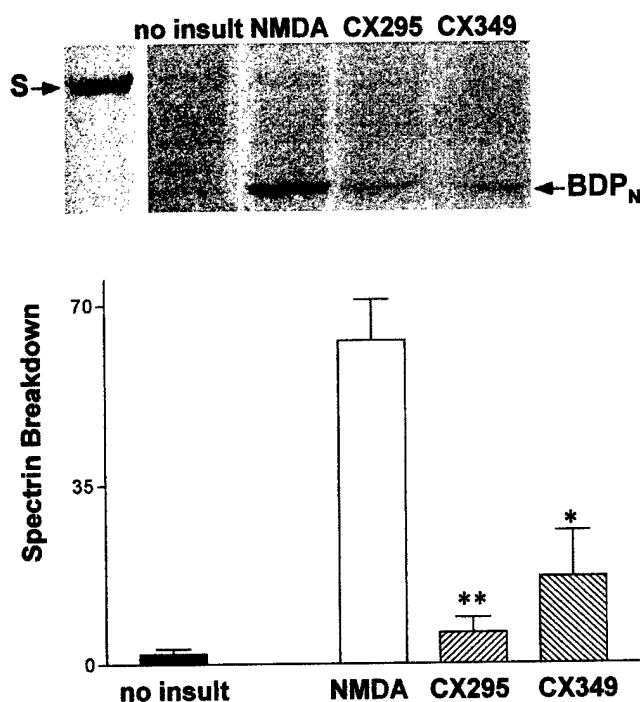
Fig. 1. Selective calpain inhibitors produce cytoskeletal protection. Cultured hippocampal slices were subjected to the 20 min NMDA insult described in Materials and Methods, with or without preincubation for 60 min with 100 μM CX295 or CX349. Slices were harvested 10 min postinsult in groups of five or six, and equal protein aliquots of the slice samples were blotted against anti-BDP$_N$, except for the first lane, which consists of control slices stained for the parent protein spectrin (S). Levels of BDP$_N$ immunoreactivity were determined by image analysis (mean ± SEM; n = 4–10 groups per condition). ANOVA: $P < 0.0001$. Post hoc Tukey test compared with NMDA insult alone: $\star P < 0.01$; $\star\star P < 0.001$.

Fig. 2. CX295 is protective when applied before or after an excitotoxic insult. Hippocampal slices were treated with different concentrations of CX295 applied before or immediately after the 20 min NMDA insult. ZLF, an inactive control compound, also was applied after the NMDA insult regimen. Groups of five or six slices each were harvested 3 hr postinsult, and equal aliquots of protein were assessed for BDP$_N$. A representative immunoblot is shown. The plotted data represent mean immunoreactivities, typically determined from n = 4–10 groups of slices (±SEM). ANOVAs were applied to the pre-CX295 data ($P < 0.0001$) and the post-CX295 data ($P = 0.0001$).

DNA–protein complexes were then resolved by 4% PAGE and visualized with Kodak X-Omat film autoradiography. Supershift analyses included incubating samples with antibodies to p50, p52, and p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min on ice before electrophoretic separation.

RESULTS

NMDA was infused into cultured hippocampal slices to produce an excitotoxic insult. Such overactivation of excitatory amino acid receptors causes cytoskeletal breakdown that leads to the degradation or truncation of synaptic components and eventual cell death (see Lee et al., 1991; Bahr et al., 1994, 2001; Gellerman et al., 1997; Bi et al., 1998). Spectrin degradation was used to monitor calpain-mediated cleavage and to confirm the neuroprotective effects of selective calpain inhibitors. To measure cytoskeletal damage, antibodies to the 150 kDa amino-terminal spectrin breakdown product BDP^N were used. As shown in the immunoblot in Figure 1, hippocampal slice cultures harvested 10 min after a 20 min NMDA insult exhibited pronounced calpain activation as indicated by production of the BDP^N species. Infusion of selective

calpain inhibitors 1 hr prior to the excitotoxic insult caused the expected decrease in proteolytic activity. The two α-keto amides, similar in structure, CX295 and CX349, caused 75–93% reduction in the NMDA-induced calpain response (Fig. 1). To test for extended protection, different concentrations of CX295 were infused before or after the 20 min NMDA insult, and the slices were subsequently harvested 3 hr postinsult. Slices preincubated with the calpain inhibitor exhibited dose-dependent cytoskeletal protection (Fig. 2; ANOVA: $P < 0.0001$). Interestingly, CX295 infused only after the excitotoxic insult also produced significant protection against cytoskeletal damage, although at a lower potency than with the pre-insult application (two-way ANOVA: $P < 0.001$). The inactive control compound Z-Leu-Phe (ZLF) had no effect on spectrin breakdown. The top panel in Figure 3 shows that, at the 3 hr postinsult time, the cytoskeletal damage was localized to dendritic fields extending from the stratum pyramidale, where the density of overactivated synapses is highest (see Bahr et al., 1994, 1995a). Cytoskeletal protection was particularly evident in the dendritic fields of stratum oriens and stratum radiatum, where the NMDA-induced BDP$_N$ staining was completely abolished by pretreatment with CX295 (Fig. 3, middle); the trace
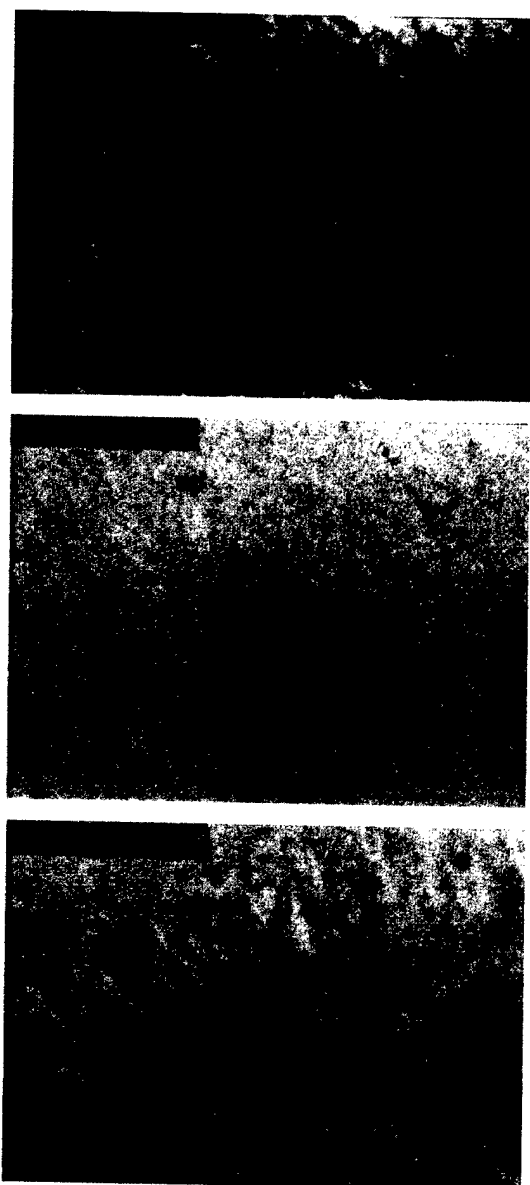
Fig. 3. Cytoskeletal breakdown in dendritic fields is reduced by CX295. Cultured slices were subjected to the NMDA insult alone (**top**) or in conjunction with preinsult (**middle**) or postinsult application of 100 μM CX295 (**bottom**), as for Figure 2. Fixed slices were sectioned at 20 μm and stained for calpain-mediated spectrin breakdown. sp, Stratum pyramidale; sr, stratum radiatum. Scale bar = 50 μm.

Fig. 4. Synaptic protection is elicited by selective calpain inhibitors. CX295 (100 μM) or CX270 (10 μM) was infused before (pre-) and after (post-) the NMDA insult. Groups of five or six slices each were harvested 3 hr postinsult and assessed by immunoblot for GluR1 and synaptophysin (Syn). Levels of immunoreactivity in n = 4–10 groups per condition are given as means ± SEM. Representative immunoblots are shown. Unpaired, two-tailed t-test compared with NMDA-treated slices without drug: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

immunolabeling remaining matched the characteristic background of noninsulted control tissue (not shown). CX295 infused postinsult also caused a marked reduction in dendritic BDP_N staining (Fig. 3, bottom).

In addition to cytoskeletal damage, excitotoxic slices exhibited corresponding synaptic deterioration, as previously indicated (Bahr, 2000; Bahr et al., 2002). For Figure 4 (bottom), the α-keto amide CX295 was preincubated to show that blockage of calpain not only produces cytoskel-

etal protection but also attenuates the loss of the presynaptic marker synaptophysin evident 3 hr postinsult. Similar presynaptic protection was produced by a member (CX270) of the α-keto acid class of calpain inhibitors (Fig. 4; ANOVA: $P < 0.001$). Infusing the selective calpain inhibitors postinsult further established the correspondence between cytoskeletal and synaptic protection. Application of CX295 before vs. after the NMDA insult promoted presynaptic maintenance by a comparable degree (post hoc Tukey tests: $P < 0.01$ and $P < 0.05$, respectively), as was the case for CX270 ($P < 0.05$ and $P < 0.01$, respectively). Similar results were found for the
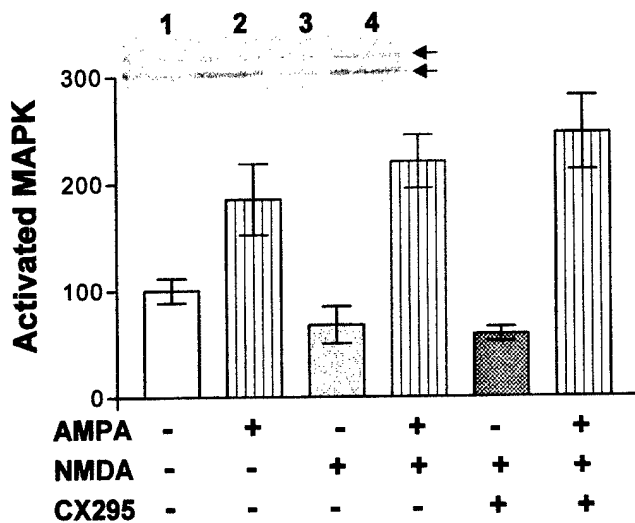
Fig. 5. Calpain inhibition by CX295 does not affect MAPK activation. Three sets of slice cultures were subjected to brief AMPA stimulation to assess for the phosphorylated active forms of ERK1 (top arrow) and ERK2 (bottom arrow). The first set consisted of control slices treated without or with 100 μM AMPA for 5 min (lanes 1 and 2, respectively), and phospho-ERK2 was quantified by image analysis (leftmost pair of bars). The other sets consisted of NMDA-treated slices in the absence (middle pair of bars) or the presence (rightmost pair of bars; lanes 3 and 4) of 100 μM CX295. The AMPA stimulation occurred 3 hr postinsult, followed by immediate harvesting into samples of five or six slices each. Means ± SEM of phospho-ERK2 levels were determined from n = 4 per condition.

two calpain inhibitors with regard to protecting against NMDA-induced modification of the postsynaptic AMPA receptor subunit GluR1 (Fig. 4, top; ANOVA: $P < 0.0001$). The loss of carboxyl-terminal staining of GluR1 represents an excitotoxic response, as shown in previous studies using cultured hippocampal slices (Bahr et al., 1994; Gellerman et al., 1997; Bi et al., 1998). Note that, in general, more presynaptic (53–80%) compared with postsynaptic protection (33–58%) was elicited by the different inhibitor applications. Synaptic protection also was evident 21 hr postinsult, where the postinsult infusion of CX295 and CX270 promoted the maintenance of synaptophysin (60% ± 5% and 73% ± 10% protection, respectively) and GluR1 structure (50% ± 9% and 59% ± 10%, respectively).

Selective calpain inhibition clearly attenuated pathogenic responses of the protease, but it is important to determine whether normal signaling events are affected. To test for the activation of the ERK1/ERK2 MAPK, AMPA was applied to the slices for 5 min, and then specific antibodies were used to measure the phosphorylated active forms of ERK1 and ERK2 (see immunoblot in Fig. 5). Brief stimulation of AMPA receptors has been shown previously to cause MAPK activation in hippocampal slice cultures through the upstream activator of MAPK kinase, also referred to as MEK (Bahr et al., 2002). Increases in phospho-ERK1 and phospho-ERK2 were not

due to an increase in total protein, as revealed by parallel blots stained for total MAPK (active and inactive ERK1/ERK2; data not shown). By measuring the levels of phospho-ERK2, the extent of AMPA-induced MAPK activation was found to be similar whether assessed before (leftmost pair of bars in Fig. 5) or 3 hr after (middle pair of bars) the 20 min NMDA insult described earlier. When CX295 was infused into the slices during the 3 hr recovery period, the α-keto amide had no effect on the basal level of phospho-ERK2 or on the AMPA-induced MAPK activation event (Fig. 5, rightmost pair of bars). As shown in the immunoblot in Figure 5, selective calpain inhibition also had no effect on the induction of activated ERK1.

CX295 also was tested for its influence on the compensatory response of NF-κB because the transcription factor appears to have a protective role in neurons (see Mattson et al., 2000) and represents a major signaling pathway for cellular maintenance. Slices were pretreated with CX295, which was continually present during the 20 min NMDA episode, the quenches, and the postinsult incubation period of 3 hr. After NMDA treatment alone, more NF-κB was translocated to the nuclear fraction of the hippocampal slice cultures and bound to its DNA consensus sequence (Fig. 6). The level of NF-κB activation was not diminished when CX295 was present for neuroprotection. In addition, the selective calpain inhibitor had no effect on the subunit composition of the activated NF-κB, as shown by the supershifts in Figure 6 using antibodies to the p50 subunit. Additional analyses indicated that the excitotoxic activation of NF-κB does not involve p52 or p65 subunits in either the absence or the presence of CX295 (not shown). These finding indicate that CX295 does not affect NF-κB activation, ruling out any interference with vital compensatory responses after an excitotoxic event.

DISCUSSION

The α-keto amide calpain inhibitor CX295 has been shown to possess sufficient potency, solubility, and membrane permeability to produce cellular and functional recovery after neurological insults (see Bartus et al., 1994b; Saatman et al., 1996). Here we tested whether CX295-mediated neuroprotection against excitotoxicity is associated with the disruption of cell signaling events, an important issue in light of recent reports that calpain is essential for certain cellular functions as well as embryonic development (Arthur et al., 2000; Zimmerman et al., 2000; Azam et al., 2001). In hippocampal slice cultures exposed to NMDA, CX295 and other selective calpain inhibitors significantly reduced the excitotoxic damage to the spectrin cytoskeleton. The levels of cytoskeletal protection achieved by blocking calpain before or after the excitotoxic insult were similar, indicating that the proteolytic response is prolonged, extending beyond the NMDA exposure period, as previously shown (Bahr et al., 1995b).

The excitotoxic activation of calpain caused pronounced and lasting spectrin breakdown in the dendritic fields of hippocampal slice cultures where glutamate receptors are concentrated (see Bahr et al., 1995a). This type
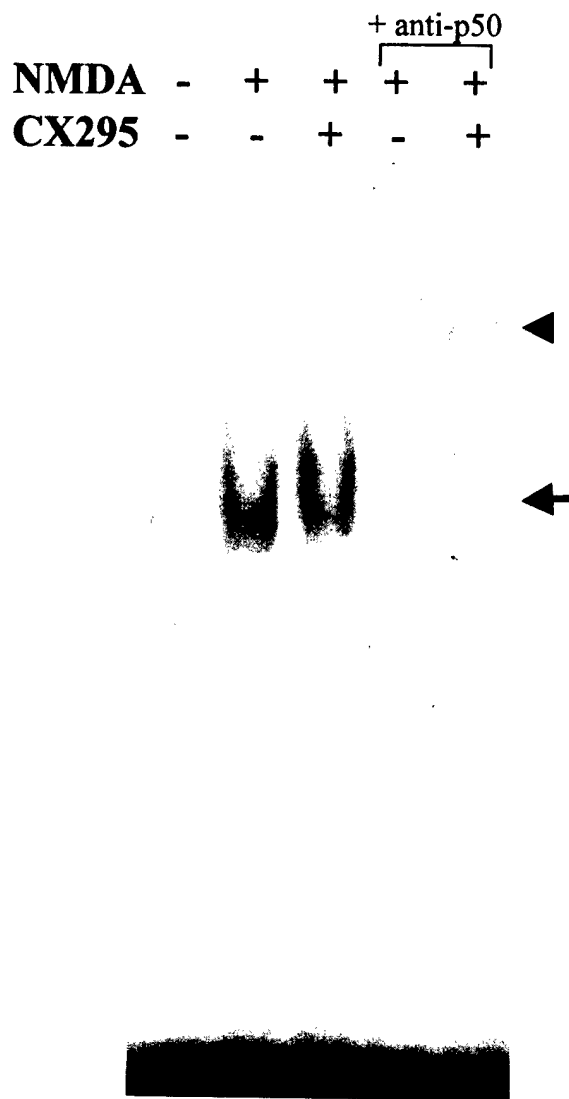
+ anti-p50

NMDA - + + + +

CX295 - - + - +

Fig. 6. NF-κB activation is not altered by CX295. Hippocampal slice cultures were subjected to NMDA excitotoxicity in the absence or presence of CX295. The slices were harvested 3 hr postinsult and then subjected to nuclear extraction to assess the activation of NF-κB. The radiolabeled band marked with an arrow consists of the consensus DNA sequence bound by NF-κB. Additional nuclear extracts were incubated with anti-p50 antibodies to test for a supershift in the activated transcription factor (arrowhead).

of proteolytic response to excitotoxic NMDA exposure has been linked to the long-term collapse of synaptic physiology (Bednarski et al., 1995) and, as shown here, to a >80% reduction in carboxyl-terminal staining of the GluR1 subunit (also see Gellerman et al., 1997). Such a correspondence between calpain-mediated spectrin cleavage and excitotoxic modification of GluR1 structure also was found in previous studies following overactivation of AMPA receptors in slice cultures (Bahr et al., 2002) and following kainic acid injection in vivo (Bi et al., 1996). In contrast, short nonpathogenic exposures to NMDA do

not elicit lasting spectrin breakdown (Bahr et al., 1995b) and cause no change or a small increase in GluR1 carboxyl-terminal staining (Broutman and Baudry, 2001). The excitotoxic changes in spectrin and GluR1 in the present study were associated with a marked decrease in synaptophysin protein levels. More importantly, calpain inhibition with CX295 nearly eliminated dendritic spectrin breakdown and, correspondingly, promoted the preservation of synaptic components. The correlation between cytoskeletal and synaptic damage in the excitotoxic slice model is in accordance with many studies linking early spectrin breakdown with subsequent cellular destruction (Vanderklish and Bahr, 2000). Thus, it is not surprising that selective calpain inhibitors cause parallel protective effects against both cytoskeletal and synaptic deterioration. Structural maintenance of the postsynaptic GluR1 subunit appeared to be enhanced by CX295 to a lesser degree than the protection elicited towards the presynaptic marker. This suggests that 1) pathogenic GluR1 modification is at least partly due to noncalpain mediated events and 2) synaptic terminals deteriorate in response to postsynaptic degeneration.

In addition to the induction of cellular damage, excitotoxic episodes also have been shown to activate MAPK and NF-κB. The kinase and the transcription factor are likely involved in compensatory mechanisms that are activated in response to injury (Shamloo et al., 1999; Mattson et al., 2000; Bahr et al., 2001). Protease inhibitors that can target calpain have been reported to block the activation of MAPK (Torres et al., 2000) and NF-κB (Chen et al., 2000; McDonald et al., 2001). In the present report, MAPK exhibited normal activation in response to brief stimulation of AMPA receptors occurring before or 3 hr after NMDA-mediated excitotoxicity. The α-keto amide CX295 had no influence on basal levels of activated MAPK isoforms, nor did the selective calpain inhibitor affect AMPA-induced kinase activation. CX295 also had no effect on the level or subunit composition of NF-κB activated in response to the NMDA exposure. Thus, although calpain has been implicated in silica-induced NF-κB activation in epithelial cells (Chen et al., 2000), the transcription factor's response to excitotoxicity in hippocampus appears to be independent of the protease.

The present results demonstrate that selective calpain inhibition elicits neuroprotection without affecting signal transduction pathways that are essential for cellular function. This is important insofar as calpain is a prominent signaling element involved in multiple pathways implicated in cytoskeletal organization (Huttenlocher et al., 1997; Kawasaki et al., 1997; Bahr, 2000), adhesion responses (Fox et al., 1993; Cooray et al., 1996; Rock et al., 1997; Bahr, 2000), intracellular signals (see Saido et al., 1994; Cooray et al., 1996), and gene regulation (Shumway et al., 1999; Chen et al., 2000; Schoonbroodt et al., 2000). To be effective, therapeutic intervention of calpain actions must not include down-regulation of compensatory responses needed for survival. In slices treated with CX295, the residual calpain-mediated proteolysis evident hours

postinsult may signify that low levels of calpain activity are somehow maintained to support signaling pathways without being pathogenic. Note that it has been shown that cytoskeletal damage by calpain must only be held below a certain threshold to promote the likelihood of cellular recovery (see Bahr et al., 2001). There are cases, however, in which complete elimination of calpain activity was found to have no effect on the survival of cultured cells (Arthur et al., 2000). In sum, selective calpain inhibitors can be used in a way that allows pathogenic proteolysis to be sufficiently reduced without disrupting important signaling pathways. This is of particular interest in the current study. Pathways involving MAPK and NF-κB may be vital for cellular maintenance systems that actually contribute to and help establish the final state of recovery.

REFERENCES

Arai A, Kessler M, Lee K, Lynch G. 1990. Calpain inhibitors improve the recovery of synaptic transmission from hypoxia in hippocampal slices. Brain Res 523:313–315.

Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA. 2000. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol Cell Biol 20:4474–4481.

Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. 2001. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. Mol Cell Biol 21:2213–2220.

Bahr BA. 1995. Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 42:294–305.

Bahr BA. 2000. Integrin-type signaling has a distinct influence on NMDA-induced cytoskeletal disassembly. J Neurosci Res 59:827–832.

Bahr BA, Arai B, Gall C, Vanderklish PW, Hoffman KB, Lynch G. 1994. Induction of β-amyloid containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 129:81–94.

Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Singh Mutneja M, Gall C, Hoffman KB. 1995a. Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5:425–439.

Bahr BA, Tiriveedhi S, Park GY, Lynch G. 1995b. Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. J Pharmacol Exp Ther 273:902–907.

Bahr BA, Hoffman KB, Kessler M, Hennegriff M, Park GY, Yamamoto RS, Kawasaki BT, Vanderklish PW, Hall RA, Lynch G. 1996. Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (GR53) in brain tissue. Neuroscience 74:707–721.

Bahr BA, Hoffman KB, Yang AJ, Hess US, Glabe CG, Lynch G. 1998. Amyloid beta protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J Comp Neurol 397:139–147.

Bahr BA, Yilma S, Suppiramaniam V. 1999. Structural chemistries underlying synaptic signaling and regulation. J Mol Biol Biotechnol 1:35–48.

Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, Rogers G. 2002. Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol (in press).

Bartus RT, Baker KL, Heiser AD, Sawyer SD, Dean RL, Elliott PJ, Straub JA. 1994a. Post-ischemic administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. J Cereb Blood Flow Metab 14:537–544.

Bartus RT, Hayward NJ, Elliott PJ, Sawyer SD, Baker KL, Dean RL,

Akiyama A, Straub JA, Harbeson SL, Li Z. 1994b. Calpain inhibitor AK295 protects neurons from focal brain ischemia. Stroke 25:2265–2270.

Bednarski E, Vanderklish PW, Gall CM, Saido TC, Bahr BA, Lynch G. 1995. Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. Brain Res 694:147–157.

Bi X, Chang V, Siman R, Tocco G, Baudry M. 1996. Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. Brain Res 726:98–108.

Bi X, Chen J, Baudry M. 1998. Calpain-mediated proteolysis of GluR1 subunits in organotypic hippocampal cultures following kainic acid treatment. Brain Res 781:355–357.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358–1362.

Brorson JR, Marcuccilli CJ, Miller RJ. 1995. Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. Stroke 26:1259–1266.

Broutman G, Baudry M. 2001. Involvement of the secretory pathway for AMPA receptors in NMDA-induced potentiation in hippocampus. J Neurosci 21:27–34.

Chan SL, Mattson MP. 1999. Caspase and calpain substrates: roles in synaptic plasticity and cell death. J Neurosci Res 58:167–190.

Chen F, Demers L, Vallyathan V, Lu Y, Castranova V, Shi X. 2000. Impairment of NF-κB activation and modulation of gene expression by calpastatin. Am J Physiol Cell Physiol 279:C709–C719.

Cooray P, Yuan Y, Schoenwaelder SM, Mitchell CA, Salem HH, Jackson SP. 1996. Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. Biochemistry 318:41–47.

DeBiasi RL, Edelstein CL, Sherry B, Tyler KL. 2001. Calpain inhibition protects against virus-induced apoptotic myocardial injury. J Virol 75:351–361.

Del Cerro S, Arai A, Kessler M, Bahr BA, Vanderklish P, Rivera S, Lynch G. 1994. Stimulation of NMDA receptors activates calpain in cultured hippocampal slices. Neurosci Lett 167:149–152.

Fox JEB, Taylor RG, Taffarel M, Boyles JK, Goll DE. 1993. Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin glycoprotein IIbIIIa. J Cell Biol 120:1501–1507.

Gellerman DM, Bi X, Baudry M. 1997. NMDA receptor-mediated regulation of AMPA receptor properties in organotypic hippocampal slice cultures. J Neurochem 69:131–136.

Han BH, Holtzman DM. 2000. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J Neurosci 20:5775–5781.

Hong SC, Goto Y, Lanzino G, Soleau S, Kassell NF, Lee KS. 1994. Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. Stroke 25:663–669.

Huttenlocher A, Palecek SP, Lu Q, Zhang W, Mellgren RL, Lauffenburger DA, Ginsberg MH, Horwitz AF. 1997. Regulation of cell migration by the calcium-dependent protease calpain. J Biol Chem 272:32719–32722.

Kako K, Wakamatsu H, Hamada T, Banasik M, Ohata K, Niki-Kuroiwa T, Suzuki S, Takeuchi J, Ishida N. 1998. Examination of DNA-binding activity of neuronal transcription factors by electrophoretical mobility shift assay. Brain Res Prot 2:243–249.

Kawasaki BT, Hoffman KB, Yamamoto RS, Bahr BA. 1997. Variants of the receptor/channel clustering molecule gephyrin in brain: distinct distribution patterns, developmental profiles, and proteolytic cleavage by calpain. J Neurosci Res 49:381–388.

Kotzbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, Johnson EM Jr, Milbrandt J. 1996. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384:467–470.

Lee KS, Frank S, Vanderklish P, Arai A, Lynch G. 1991. Inhibition of proteolysis protects hippocampal neurons from ischemia. Proc Natl Acad Sci USA 88:7233–7237.

Li Z, Patil GS, Golubski ZE, Hori H, Tehrani K, Foreman JE, Eveleth DD, Bartus RT, Powers JC. 1993. Peptide α-keto ester, α-keto amide, and α-keto acid inhibitors of calpains and other cysteine proteases. J Med Chem 36:3472–3480.

Li Z, Ortega-Vilain AC, Patil GS, Chu DL, Foreman JE, Eveleth DD, Powers JC. 1996. Novel peptidyl α-keto amide inhibitors of calpains and other cysteine proteases. J Med Chem 39:4089–4098.

Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD. 1998. Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke 29:152–158.

Mattson MP, Culmsee C, Yu ZF, Canandola S. 2000. Roles of nuclear factor κB in neuronal survival and plasticity. J Neurochem 74:443–456.

McDonald MC, Mota-Filipe H, Paul A, Cuzzocrea S, Abdelrahman M, Harwood S, Plevin R, Chatterjee PK, Yaqoob MM, Thiemermann C. 2001. Calpain inhibitor I reduces the activation of nuclear factor-kappaB an organ injury/dysfunction in hemorrhagic shock. FASEB J 15:171–186.

Neumar RW, Meng FH, Mills AM, Xu YA, Zhang C, Welsh FA, Siman R. 2001. Calpain activity in the rat brain after transient forebrain ischemia. Exp Neurol 170:27–35.

Posmantur R, Kampfl A, Siman R, Liu J, Zhao X, Clifton GL, Hayes RL. 1997. A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat. Neuroscience 77:875–888.

Rami A, Krieglstein J. 1993. Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia in vitro and ischemia in vivo. Brain Res 609:67–70.

Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. 1994. Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. J Neurosci 14:3934–3944.

Rock MT, Brooks WH, Roszman TL. 1997. Calcium-dependent signaling pathways in T cells. Potential role of calpain, protein tyrosine phosphatase 1b, and p130Cas in integrin-mediated signaling events. J Biol Chem 272:33377–33383.

Saatman KE, Murai H, Bartus RT, Smith DH, Hayward NJ, Perri BR, McIntosh TK. 1996. Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. Proc Natl Acad Sci USA 93:3428–3433.

Saido TC, Yokota M, Nagao S, Yamaura I, Tani E, Tsuchiya T, Suzuki K, Kawashima S. 1993. Spatial resolution of fodrin proteolysis in postischemic brain. J Biol Chem 268:25239–25243.

Saido TC, Sorimachi H, Suzuki K. 1994. Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J 8:814–822.

Santella L, Kyozuka K, Deiso L, Carafoli E. 1998. Calcium, protease action, and the regulation of cell cycle. Cell Calcium 2-3:123–130.

Schoonbroodt S, Ferreira V, Best-Belpomme M, Boelaert JR, Legrand-Poels S, Korner M, Piette J. 2000. Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl terminal PEST domain of IκBα in NF-κB activation by an oxidative stress. J Immunol 164:4292–4300.

Schreiber E, Matthias P, Muller MM, Schaffner W. 1989. Rapid detection of octamer binding proteins with "mini-extracts," prepared from a small number of cells. Nucleic Acids Res 17:6419.

Shamloo M, Rytter A, Wieloch T. 1999. Activation of the extracellular signal-regulated protein kinase cascade in the hippocampal CA1 region in a rat model of global cerebral ischemic preconditioning. Neuroscience 93:81–88.

Shumway SD, Masatoshi M, Miyamoto S. 1999. The PEST domain of IκBα is necessary and sufficient for in vitro degradation by μ-calpain. J Biol Chem 274:30874–30881.

Torres C, Li M, Walter R, Sierra F. 2000. Modulation of the ERK pathway of signal transduction by cysteine protease inhibitors. J Cell Biochem 80:11–23.

Utz PJ, Anderson P. 2000. Life and death decisions: regulation of apoptosis by proteolysis of signaling molecules. Cell Death Differ 7:589–602

Vanderklish PW, Bahr BA. 2000. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 81:323–339.

Villa PG, Henzel WJ, Sensenbrenner M, Henderson CE, Pettmann B. 1998. Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. J Cell Sci 111:713–722.

Yokota M, Tani E, Tsubuki S, Yamaura I, Nakagaki I, Hori S, Saido TC. 1999. Calpain inhibitor entrapped in liposome rescues ischemic neuronal damage. Brain Res 819:8–14.

Zimmerman UJ, Boring L, Pak JH, Mukerjee N, Wang KK. 2000. The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life 50:63–68.

Master

Intracellular Deposition, Microtubule Destabilization, and Transport Failure: An "Early" Pathogenic Cascade Leading to Synaptic Decline

JENNIFER BENDISKE, PhD, EBRU CABA, MS, QUEENIE B. BROWN, MS, AND BEN A. BAHR, PhD

Abstract. Protein deposition is a common event in age-related neurological diseases that are characterized by neuronal dysfunction and eventual cell death. Here, cultured hippocampal slices were infused with the lysosomal disruptor chloroquine to examine the link between abnormal protein processing/deposition and early synaptopathogenesis. Tau species of 55 to 69 kDa increased over several days of treatment with chloroquine, while the protein and message levels of synaptic markers were selectively reduced. Neurons of subfields CA1, CA3, and dentate gyrus accumulated protein deposits recognized by antibodies against paired helical filaments and ubiquitin, which was accompanied by tubulin fragmentation and deacetylation. The deposition filled the basal pole of pyramidal neurons, encompassing the area of the axon hillock and initial dendritic branching but without causing overt neuronal atrophy. Neurons containing the polar aggregates exhibited severely impaired transport along basal dendrites. Transport capability was also lost along apical dendrites, the opposite direction of deposited material in the basal pole; thus, perpetuating the problem beyond physical blockage must be the associated loss of microtubule integrity. These data indicate that transport failure forms a link between tau deposition and synaptic decline, thus shedding light on how protein aggregation events disrupt synaptic and cognitive functions before the ensuing cellular destruction.

Key Words: Acetylated tubulin; Alzheimer disease; Hippocampal slice culture; Neurodegeneration; Paired helical filaments; Tau.

INTRODUCTION

Intracellular deposition is common among several age-related neurodegenerative disorders, including Alzheimer disease (AD) (1), Huntington disease (2, 3), and α-synuclein disorders such as frontotemporal dementia and Parkinson disease (1, 4). Also common among such disorders is evidence of disrupted protein processing pathways in lysosomes (5–7). In Alzheimer brains, in particular, lysosomal disturbances have been implicated in the formation of amyloidogenic fragments of the amyloid precursor protein (8–10). The carboxyterminal fragments are deposited in intracellular compartments that are consistent with lysosomes (11–15). In vivo (16) and in vitro studies (12, 17, 18) confirmed that experimentally-induced lysosomal disruption leads to the production of β-amyloid-bearing polypeptides.

Lysosomal dysfunction also has been linked to alterations in the microtubule-binding protein tau and the subsequent intraneuronal deposition of hyperphosphorylated isoforms in the form of paired helical filaments (PHFs) (19–23). PHF deposits facilitate production of neurofibrillary tangles (NFTs) in brain areas targeted by AD (24–27), and the degree to which vulnerable regions express neurodegeneration appears to be determined by their relative content of tau isoforms capable of transforming into PHFs (28). In addition to being an indicator

of advanced stages of the disease (29, 30), NFT deposition has been correlated with diminished expression of genes encoding the presynaptic proteins synaptophysin and GAP43 (31). Synaptophysin mRNA levels indicate the amount of synaptophysin protein located at synaptic terminals (32), and a decline in this protein is indicative of synaptic deterioration in Alzheimer tissue (33–35).

Besides a role in amyloidogenic chemistries and tau modifications, lysosomal dysfunction leads to synaptic decline demonstrated by 1) a marked loss of presynaptic proteins, 2) delayed reductions in postsynaptic components, and 3) concomitant disruption of neuronal communication (12). While many studies have found correlations of different degrees between protein deposition and the severity of neurodegenerative disorders, how abnormal protein processing events facilitate the deterioration of synaptic structure and functionality is unknown. In order to approach this question, cultured hippocampal slices were exposed to chloroquine, a lysosomal disruptor that inhibits protein degradation in neurons by elevating the pH of lysosomes (36–38). Using the slice model, we report here corresponding events of i) polar deposition of material labeled by antibodies against human PHFs, ii) microtubule destabilization and tubulin breakdown, iii) somatofugal transport failure, and iv) synaptic decline at both the protein and message level. The results support the hypothesis that abnormal protein processing/deposition contributes to early synaptic deterioration by disrupting microtubule-based transport processes that are vital for synapse maintenance.

MATERIALS AND METHODS

Antibodies and Chemicals

Monoclonal antibodies against synaptophysin, α-acetylated tubulin, and tau-1 were obtained from Chemicon (Temecula,
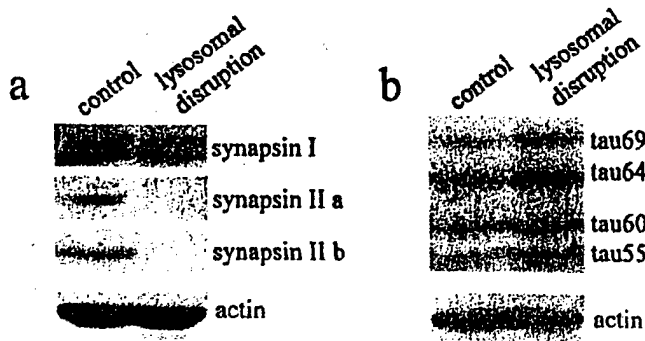
Fig. 1. Lysosomal dysfunction leads to reductions in synapsin proteins and increases in tau isoforms. Chloroquine (60 μM) was administered for 3 to 6 days in order to induce lysosomal disruption in hippocampal slices. The slices were harvested in groups of 6 to 8 and prepared for immunoblot analysis. The typical immunoblots shown were obtained from sample lysates probed for synapsin I, IIa, and IIb, (a) and tau species (b). Actin was assessed on the same blot that was stained for synapsin IIa and IIb in panel (a), and the same blot stained for tau in panel (b). Unchanged actin levels were found on all blots tested. Prior to staining for tau isoforms, blots were treated with alkaline phosphatase as described in Materials and Methods.
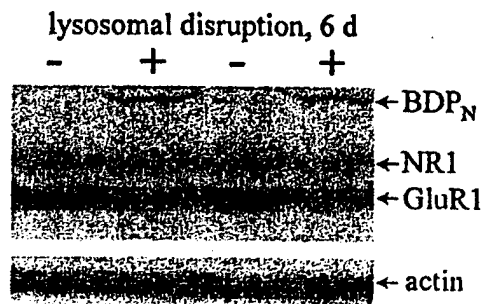
Fig. 2. Secondary pathology of cytoskeletal breakdown accompanies the synaptic decline in chloroquine-treated slices. Lysosomal dysfunction was induced for 6 days, after which the cultured hippocampal slices were prepared for immunoblot analysis in groups of 6 to 8. The blot shown was probed consecutively for calpain-mediated BDP_N, GluR1, NR1, and actin.

CA), ICN Biomedicals, Inc. (Costa Mesa, CA), and Boehringer Mannheim (Indianapolis, IN), respectively. Polyclonal antibodies were obtained as follows. Antibodies to synapsin I and II were from Calbiochem (San Diego, CA), PHF-1 antibodies from ICN Biomedicals, anti-actin from Sigma Chemical Company (St. Louis, MO), anti-ubiquitin from Dako Corporation (Glostrup, Denmark), and antibodies to the NR1 subunit from Chemicon. Affinity-purified antibodies to the AMPA receptor subunit GluR1 were prepared as described (39). The cytoskeletal breakdown product BDP_N was measured using affinity-purified antibodies against end residues (Gln-Gln-Glu-Val-Tyr) of the aminoterminal spectrin fragment produced by calpain I (70). Millicell-CM culture inserts with Biopore membranes were from Millipore Corporation (Bedford, MA). All other cell culture supplies, chloroquine, and horseradish peroxidase were obtained from Sigma Chemical Company. Protease inhibitors such
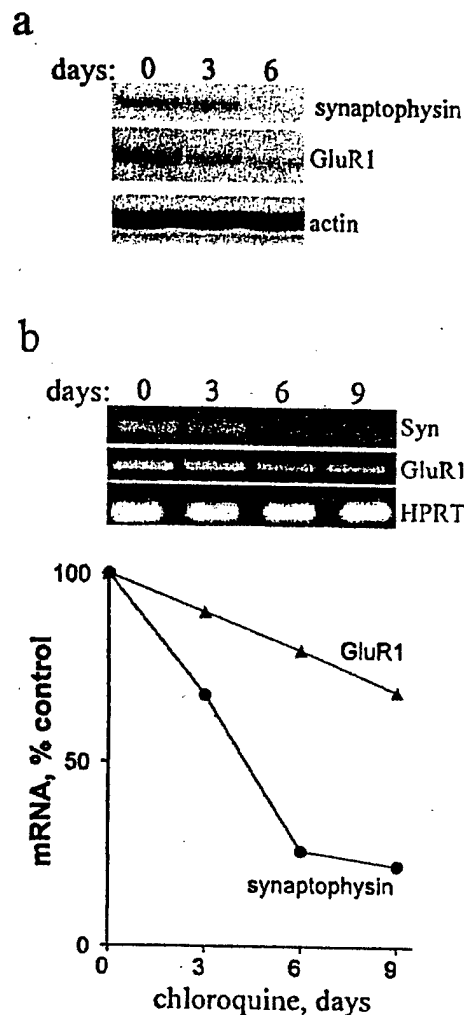

Fig. 3. Decreases in synaptic protein and message levels occur over time of chloroquine infusion. Cultured slices were treated with the lysosomal inhibitor for the days indicated, and then harvested in groups of 6 to 8 for immunoblot analyses or 20 to 40 for RT-PCR. Actin was assessed on the same immunoblot stained for synaptophysin (Syn) and the AMPA receptor subunit GluR1 (a). The synaptic mRNAs and the HPRT control message were amplified from the same RNA preparations (b). RT-PCR products were quantified by image analysis and plotted as percent of non-treated control values.

as aprotinin and pepstatin A were from Boehringer Mannheim. Nitrocellulose paper was from Osmonics, Inc. (Westborough, MA). Alkaline phosphatase-conjugated and horseradish peroxidase (HRP)-conjugated antibodies and substrate kits were from Bio-Rad Laboratories (Richmond, CA) and Vector Laboratories Inc. (Burlingame, CA).

Organotypic Hippocampal Slice Cultures and Treatment

Conventional methods were used to prepare hippocampal slices from 11- to 12-day postnatal Sprague-Dawley rat pups (Charles River Laboratories, Wilmington, MA). The animals were killed by isoflurane anesthesia (Baxter, Deerfield, IL) and decapitation. Transverse slices of hippocampus (400 μm) were
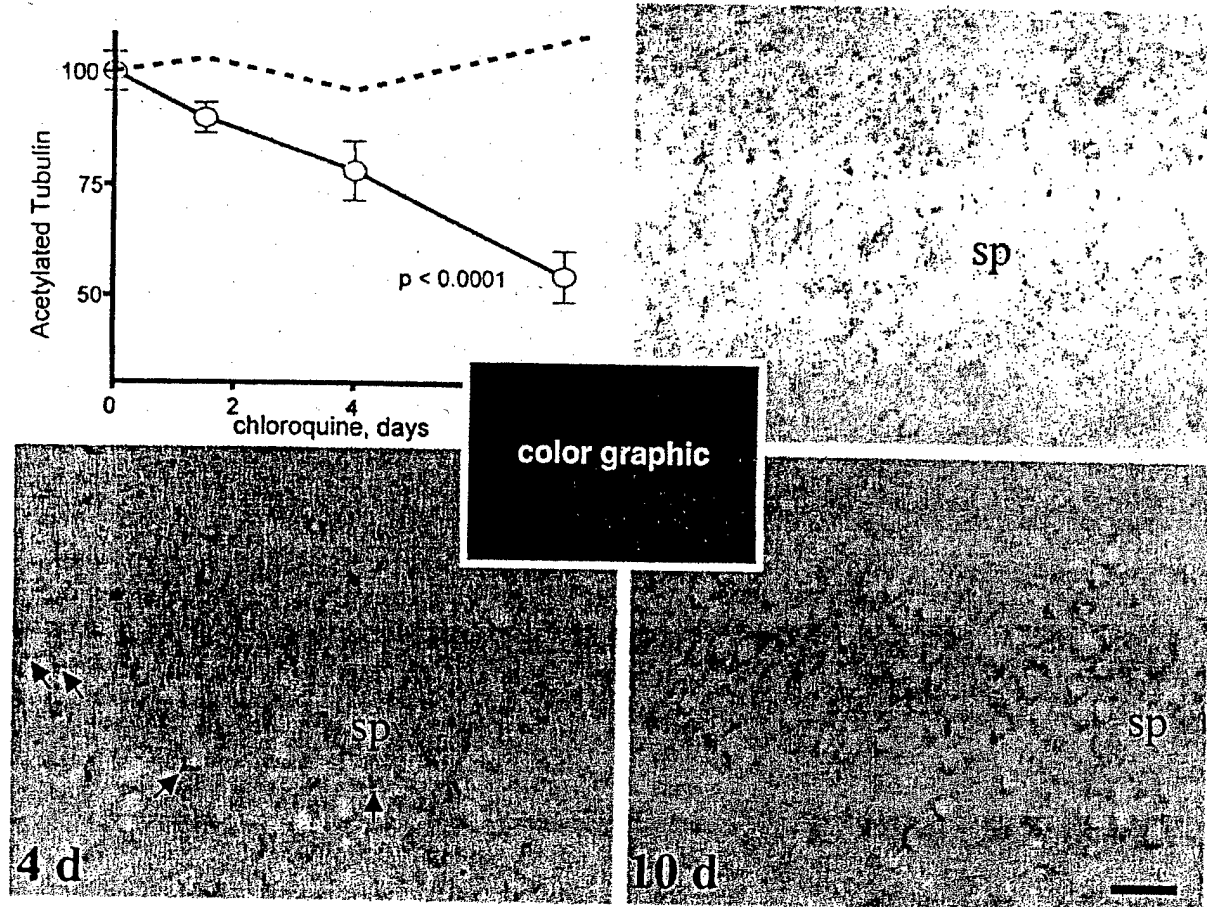
Fig. 4. Tubulin deacetylation correlates with intracellular protein deposition. Upper left graph: Hippocampal slice cultures treated with chloroquine (60 μM) for the indicated time period were analyzed by immunoblot for acetylated tubulin (open circles) and total tubulin content (dotted line); both were plotted as percent of non-treated control values. Mean immunoreactivities for acetylated tubulin were determined from 4 to 7 groups of slices (±SEM) and were assessed by ANOVA (p < 0.0001). Histology panels: Cultured slices treated with chloroquine for 1, 4, or 10 days were fixed, sectioned, and examined for PHF-1 immunoreactivity in field CA3. The photomicrographs indicate that intracellular deposition in pyramidal neurons is first apparent after 4 days of treatment (arrows). sp, stratum pyramidale; Scale bar: 45 μm.

quickly prepared from precooled hippocampi that were incubated in ice-cold buffer containing (in mM) 124 NaCl, 3 KCl, 2 CaCl₂4 MgSO₄, 1.25 KH₂PO₄, 26 NaHCO₃, 10 D-glucose, and 2 ascorbic acid. Groups of 8 to 10 slices were placed on insert membranes that were in contact with culture medium composed of 50% basal medium Eagle, 25% Earle's balanced salts, 25% horse serum, and defined supplements (21). Slices were maintained at 37°C with a 5% CO₂-enriched atmosphere and fed every other day until use.

After 10 to 15 days in culture, the slices were incubated with media containing 60 μM chloroquine or vehicle, which was changed every 2 days. Treatments were staggered so that all slices were in culture for the same length of time. Chloroquine is a weak base that accumulates in acidic lysosomes by permeation and proton trapping (58). The lysosomotropic agent disrupts protein degradation (36–38, 59), especially the degradation processes mediated by the lysosomal hydrolase cathepsin B (38), while having no direct effect on synthesis (59). Slice treatments used the 60-μM dose of chloroquine since 50 to 60

μM was the optimal range shown previously to influence lysosomal processes without affecting protein glycosylation, tyrosine sulfation, protein synthesis and secretion, or general neuronal properties underlying the waveform of synaptic and antidromic responses (12, 36, 59). The characteristic delayed effect of chloroquine on protein processing and synaptic composition is nearly identical to that produced by 1) inhibiting specific cathepsins (12, 21, 23), 2) by the broad cysteine protease inhibitor leupeptin (12, 19–22), and 3) by an ATPase blocker that also affects lysosomal pH (12).

Immunoblot Analysis

Cultured slices were gently removed with a soft brush and homogenized in groups of 6 to 8 slices each using ice-cold homogenization buffer. Protein content was determined with a BSA standard and equal protein aliquots of the slice samples were denatured in sampling buffer for 5 min at 100°C, then separated by 4% to 16% SDS-PAGE and blotted to nitrocellulose. Secondary antibody incubation utilized anti-IgG-alkaline
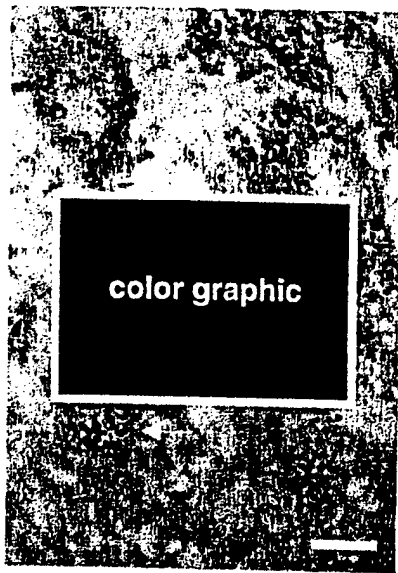
color graphic

Fig. 5. Chloroquine causes early deposition of PHF-immunopositive inclusions in CA1. Cultured slices treated with chloroquine for 4 days were fixed, sectioned, and stained with PHF-1 antibodies. The sections were subjected to digital deconvolution analysis at 1-μm intervals to obtain a stacked image showing inclusions in the basal pole of pyramidal neurons (arrows). Scale bar: 17 μm.

phosphatase conjugates, and color development used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Bands were scanned at high resolution and assessed for integrated density with BIOQUANT software (R & μM Biometrics, Nashville, TN). Prior to staining for tau-1 immunoreactivity, the nitrocellulose blots were treated with alkaline phosphatase (AP) at a concentration of 0.1 units/ml in 5% BSA (8.3). The blots were incubated with the AP solution overnight at 37°C with mild agitation. Subsequent to the incubation, the AP was removed and the blots washed. Tau-1 staining then proceeded as indicated above.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cultured slices were harvested and RNA was isolated using RNase Easy Mini kit (Quiagen, Valencia, CA). cDNA was generated in a total volume of 20 μl using 2.5 μM random hexamers, 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 1 mM of each dNTP, 1 U/μl RNase inhibitor, and 2.5 U/μl MuLV reverse transcriptase obtained from Applied Biosystems (Branchburg, NJ). The PCR was carried out using 200 ng cDNA in a total volume of 25 μL containing 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM forward and reverse primers, and 1.25 U/μl Taq DNA polymerase. Synaptophysin, GluR1, and housekeeping gene (HPRT) primers were designed according to the GenBank sequence data bank for the respective genes, and synthesized by the University of Connecticut Biotechnology Center. The PCR products were electrophoretically

separated on 2% agarose gels and their images captured and analyzed using the Kodak EDAS120 system (Rochester, NY).

Immunocytochemistry

The cultured slices were rinsed with 0.1 M phosphate buffer (PB), pH 7.4, and fixed for 2 hours (h) in cold PB plus 4% paraformaldehyde. Slices were then cryoprotected and carefully removed from the insert, and serial sections were cut at 20-μm thicknesses and mounted on Superfrost plus coated slides (Fisher Scientific, Pittsburgh, PA). Sections were immunolabeled with PHF-1 and anti-ubiquitin antibodies using the avidin-biotin-peroxidase technique and 3,3'-diaminobenzidine as the chromogen. Localization of immunostained deposits was assessed with regards to whether the material encompassed the cellular pole that contains the axon hillock. Some sections were Nissl stained. Image analysis included the use of the computerized deconvolution Openlab system with motorized Z focus drive (Improvision, Lexington, MA).

Transport Analyses

A semi-dry slurry was prepared with 20 mg of HRP in slice buffer containing 2 μM sucrose. While still in contact with the membrane inserts, a small amount of the prepared slurry was administered to pyramidal neurons of control and chloroquine-treated hippocampal slices via a fine-tip electrode. After 2 h at 37°C the treated slices were washed in PB and then fixed in cold PB plus 4% paraformaldehyde for 2 h. Slices were then cryoprotected in a 20% sucrose solution overnight, carefully removed from the inserts, and mounted on gelatin-coated slides. The slices were then stained for 5 to 10 min with a peroxidase substrate kit containing 3-3'-diaminobenzidine and washed in water. Subsequent to color development, slices were rapidly dehydrated through ascending concentrations of ethanol, cleared in AmeriClear (Baxter), and coverslipped with Permount.

RESULTS

Cultured hippocampal slices were infused with the acidotropic agent chloroquine in order to induce gradual perturbation of lysosomal activity. The lysosomal disturbance caused marked reductions in the levels of synapse-related proteins (Figs. 1a, 2, 3a) and mRNA species (Fig. 3b). Similar to the level of reduction reported for synaptophysin (12), synapsin Ia/Ib (Fig. 1a), IIa, and IIb exhibited 30% to 50% declines after 3 days of chloroquine exposure and, in most cases, almost complete loss after 6 days. Figure 3b shows that expression of the synaptophysin gene is down regulated before the reduction in expression of AMPA-type glutamate receptor subunit GluR1. The protein reductions were synapse-specific in nature as indicated by the stable levels of actin (Figs. 1, 2) and tubulin (Fig. 4). In addition, previous work showed that lysosomal dysfunction has no effect on other cytoskeletal components or cellular morphology, while synaptic markers decreased in concentration (12). At 6 days of lysosomal disruption, initial signs of secondary pathology were evident as indicated by the activation of the protease calpain (Fig. 2). Calpain-mediated spectrin

breakdown product (BDP_N) is a sensitive measure of pathogenic events (21), and its production was pronounced in chloroquine-treated immunoblot samples (mean integrated density $\pm$SEM: 45 $\pm$ 2, n = 9) as compared to trace levels found in control slices (integrated density <2). Pathogenic calpain activation occurred in slice samples exhibiting significant reductions in the GluR1 subunit and in the NMDA receptor subunit NR1 (61% $\pm$ 6% and 59% $\pm$ 7% decreases, respectively; n = 9, p < 0.0001 each). GluR1 is also a substrate for calpain while NR1 is not (40).

In accordance with the synaptic decline, protein deposition also developed in a gradual manner during the lysosomal disturbance period. As shown in the photomicrographs of Figure 4, antibodies to human PHFs (PHF-1) recognized deposits in pyramidal neurons after 4 days of chloroquine infusion (see arrows). The intracellular deposition intensified until all neurons contained an aggregated mass of material after 10 days of treatment with the lysosomal disruptor. The deposited material presumably is composed of phosphorylated tau species of 55 to 69 kDa that, as a group, exhibited a chloroquine-induced increase in integrated density from 98 $\pm$ 43 to 279 $\pm$ 81 (Fig. 1b; p < 0.02) and shifted to smaller sizes after phosphatase treatment (not shown). Increases in similar tau species have been reported in the aged mouse brain (28, 41) and in AD (42, 43). With deconvolution and image stacking, the early deposition in CA1 neurons was shown to be comprised of PHF-immunoreactive inclusions (Fig. 5, arrows).

The induced alteration/deposition of microtubule-binding tau species was associated with changes in microtubule chemistries. In Figure 6, hippocampal slice cultures were treated with chloroquine for 0 to 8 days then assessed for tubulin acetylation by staining blot samples with antibodies that selectively recognize the acetylated form of the microtubule subunit (Ac-TN). Acetylation of tubulin is a post-translational modification that provides a marker for determining the extent of microtubule stability (44). As shown, acetylated tubulin decreased gradually over time after addition of the lysosomal disruptor. The reduction was specific for the acetylated form since total tubulin was unchanged during the lysosomal disturbance period (Figs. 4 [graph], 6 [top]). Microtubule destabilization was further indicated by evidence of fragmentation events that target acetylated tubulin. The immunoblot in Figure 6 shows that antibodies to acetylated tubulin labeled fragments of approximately 30 and 40 kDa (see arrows), and production of these fragments was gradually induced by the chloroquine treatment. The smaller fragment appears to be the more stable of the two.

The gradual loss of acetylated tubulin and the slow production of related fragments are in close correspondence with the time course of intracellular deposition in

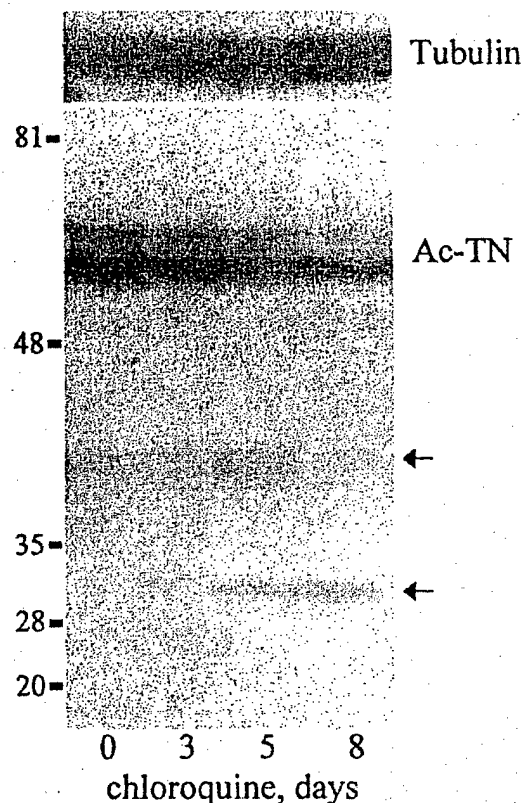
Tubulin

Ac-TN

0   3   5   8
chloroquine, days

Fig. 6. Lysosomal dysfunction induces signs of microtubule destabilization. Cultured slices were treated with chloroquine, then homogenized and separated via SDS-PAGE for immunoblot detection of total tubulin (top) and acetylated tubulin (Ac-TN). Arrows denote the location of fragments detected by affinity-purified antibodies against the acetylated form of tubulin. The electrophoretic positions of 20- to 81-kDa standards are shown on the left.

slices subjected to lysosomal disturbance (Figs. 4, 6). Material labeled by PHF-1 antibodies began to deposit in neurons after 3 to 4 days of chloroquine exposure, the same time at which signs of microtubule destabilization and synaptic decline first occurred. Linear regression was conducted on the integrated density values for acetylated tubulin and synaptophysin from individual blot samples. As acetylated tubulin decreased with different chloroquine treatment times, there was a correlated reduction in synaptophysin protein levels across the slice samples (r = 0.76, p < 0.0001), thus indicating a link between microtubule destabilization and synaptic decline (Fig. 7). No corresponding changes were found after only 1 day of chloroquine treatment, that is, PHF-1 antibodies did not label intracellular deposits, acetylated tubulin levels were not significantly altered, tubulin fragmentation events did not occur, and, as previously shown (12), the initiation of synaptic decline was not evident.

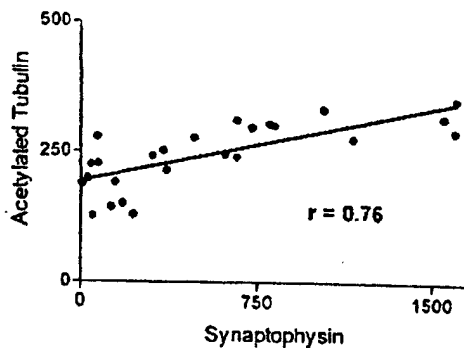The deposited material resulting from lysosomal dysfunction not only contained PHF epitopes but also was

Fig. 7. Concentrations of acetylated tubulin correlate with synaptophysin levels. Linear regression analysis was conducted on the immunoreactivity measures of acetylated tubulin and synaptophysin from individual blot samples of the plotted study shown in Figure 4. The obtained correlation coefficient was 0.76 (p < 0.0001, 1-sample t-test of slope).

ubiquitinated. Anti-ubiquitin antibodies intensely labeled deposits that gradually accumulated in pyramidal neurons over time of chloroquine treatment (Fig. 8b). Control tissue over-stained with the same antibodies showed that while background staining increased, no intracellular deposits were detected (Fig. 8a). Similar ubiquitinated deposition was elicited by treating slices for 6 days with 60 μM deacetyl-leupeptin or 100 μM E-64, two protease inhibitors known to block a number of lysosomal enzymes (data not shown). The globular mass induced by chloroquine resembled the material recognized by antibodies to human PHFs (Fig. 4). Using both antibodies, chloroquine-mediated deposition was identified in pyramidal neurons of fields CA1 (Fig. 8b) and CA3 (Fig. 4), as well as in dentate gyrus granule cells (Fig. 8c, d). The intracellular deposition occurred in a polar manner, building up exclusively in 1 pole of the soma to encompass the area of the axon hillock and initial branching of basal dendrites in pyramidal neurons. As illustrated in Figure 9, the polar accumulation coincides with the area from which axons (drawn as blue lines) stem from neurons of the 3 hippocampal subfields. Despite the pronounced accumulation of material after ≥6 days of chloroquine treatment, there were no overt signs of cell death (Fig. 10c).

The polar nature of the chloroquine-induced deposition strongly suggests that cellular transport along basal dendrites of the stratum oriens would be physically obstructed, while apical dendrites of the stratum radiatum (sr) (Fig. 9) would maintain transport capability. To test whether the polar deposition is associated with such unidirectional blockage, HRP was applied to a small zone of the s. pyramidale (Fig. 9, see boxed area) and the peroxidase activity was assessed 2 h later. In a control slice viewed by dark-field microscopy, HRP internalized by CA1 neurons was subsequently transported into the elaborate arborization of basal and apical dendrites (Fig.

10a). High-power light-field micrographs indicated that HRP extended into distal dendrite branches, spine structures, and long axons (not shown). Such transport was greatly diminished by the effects of the lysosomal disruptor chloroquine. After 6 days of chloroquine exposure, HRP transport into basal dendrites of the stratum oriens was completely blocked despite obvious perikaryontic uptake (Fig. 10b).

While transport failure in the basal direction was expected in light of localized protein deposition, it was surprising to find only trace indications of transport along apical dendrites (Fig. 10b, arrows). Thus, lysosomal dysfunction leads to widespread transport failure, with at least 1 direction being blocked independent of the physical obstruction. Similar to that found in field CA1, transport was disrupted in both dendritic directions in CA3. When chloroquine-treated tissue was stained with the tau-1 antibody, the microtubule-binding protein was found concentrated in the proximal area of apical dendrites (Fig. 11b) instead of the characteristic diffuse distribution in control tissue (Fig. 11a). Such evidence of altered tau and its disrupted transport also was found when slices were treated with cathepsin inhibitors that cause amyloidogenic fragment buildup and synaptic decline (12, 21) (data not shown). Slice cultures treated for 1 day with chloroquine exhibited normal HRP transport along apical dendrites (n = 17 slices), similar to that found in 82 control slices. After 3 and 5 days of lysosomal dysfunction, however, 4 of 22 (18%) and 17 of 35 slices (49%) exhibited severely impaired transport, respectively. Following 8 days of exposure, HRP transport was severely impaired in 48 of 68 slices (70%) and impaired at the moderate/severe level in 63 of the 68 slices (92%). Thus, transport processes were gradually impaired over days of chloroquine treatment, similar to the gradual nature of intracellular deposition, deacetylation of tubulin, tubulin fragmentation, and synaptic decline. Interestingly, these deleterious events and resulting transport failure occurred in neurons that were intact and with no evident pyknotic changes (Fig. 10c). The neuronal density in chloroquine-treated slices was consistent with that found in control slice cultures.

DISCUSSION

The present study shows that in addition to promoting amyloidogenic accumulation (12), suppression of lysosomal activity also leads to changes in tau and related deposition of PHF-immunopositive material in hippocampal neurons. Such events of abnormal protein processing were linked to the selective decline in expression of synaptic proteins including synapsin isoforms and synaptophysin. Similar declines have been shown to accompany early clinical stages of AD, especially in the hippocampus (45-48). Previous studies found these types of reductions to be indicative of synaptic decay (33, 49, 50),
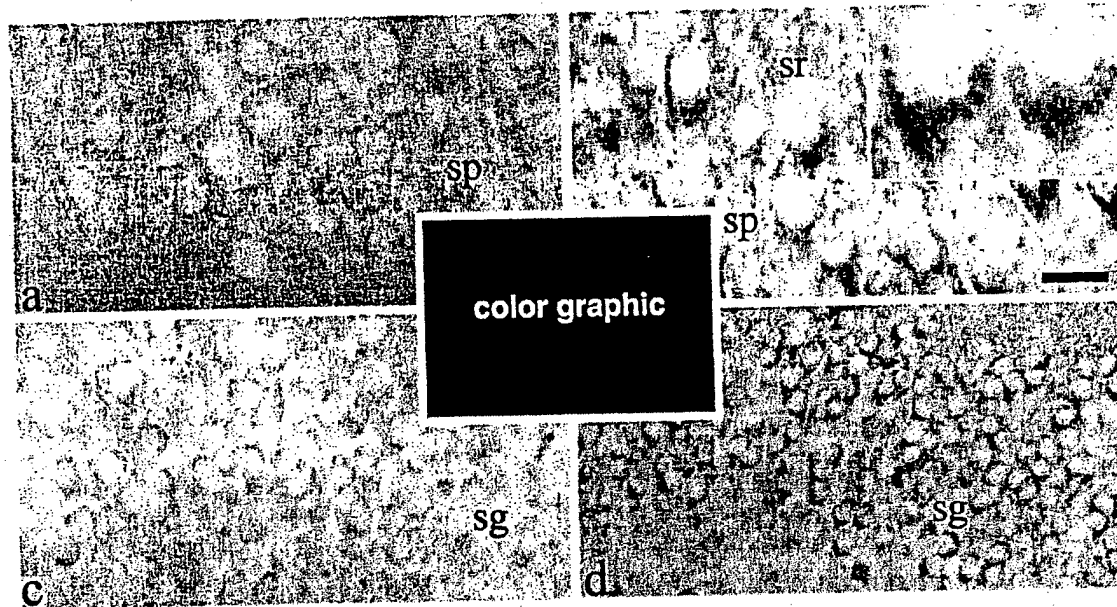
Fig. 8. Chloroquine induces polar deposits recognized by antibodies to PHFs and ubiquitin. Sections from control (a and c) and chloroquine-treated tissue (10 day treatment; b and d) were immunostained with antibodies against ubiquitin (a and b) and PHF-1 (c and d). The inset in (b) shows a higher magnification view of chloroquine-treated pyramidal neurons. Abbreviations: sp, stratum pyramidale; sg, stratum granulosum; sr, stratum radiatum. Scale bars: a = 30 μm; b = 25 μm; b inset = 9 μm; c = 30 μm; d = 35 μm.
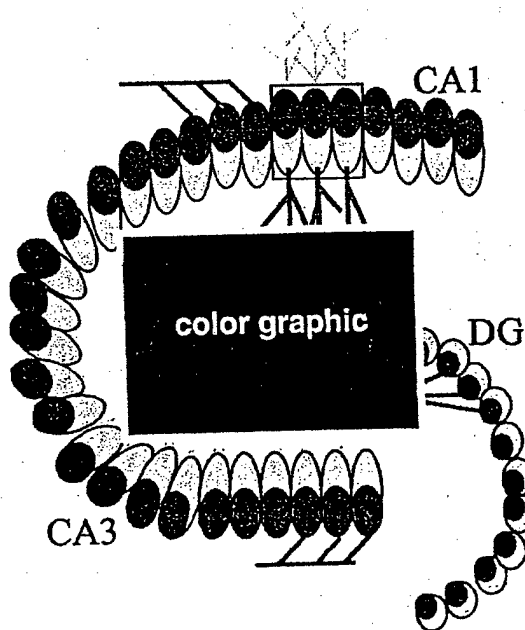
Fig. 9. Diagram of intracellular deposition. The polar deposition induced by chloroquine is shown in brown. Axons are shown in blue and the yellow and green extensions portray the basal and apical dendrites, respectively. Abbreviations: DG, dentate gyrus; sr, stratum radiatum.

leading to the identification of synapse loss as a major correlate of cognitive decline (35, 51–53).

The link between synaptic decline and intracellular deposition found in the hippocampal slice model is similar to that identified in Alzheimer tissue (31, 35, 46). Protein deposition has been shown to correlate with the severity of neurodegenerative dementias (1), thus explaining the connection between synaptic decline and cognitive impairment. However, little is known pertaining to how intracellular protein aggregates disrupt synaptic mechanisms. The present results show that experimentally induced lysosomal dysfunction down-regulates the expression of synaptic proteins, and this corresponds with the generation of intraneuronal aggregates throughout the hippocampal subregions. Similar to the NFTs found in Alzheimer brains, the deposited material was recognized by antibodies to human PHFs and to ubiquitin. These immunopositive inclusions deposited early in the slice model in a pattern reminiscent of organelles, a finding that is consistent with reports indicating that PHF formation involves lysosomes (54, 55). PHF-tau also forms in Niemann-Pick type C disease where lysosomal dysfunction results from a single gene mutation (56). In the Alzheimer hippocampus, elevated levels of cathepsin D, an indicator of lysosomal stress, and reduced expression of synaptic mRNAs were found in tangle-bearing pyramidal neurons but not in neighboring NFT-free neurons (31, 46) Lysosomes also are key organelles in the very early pathogenesis of prion encephalopathies, another
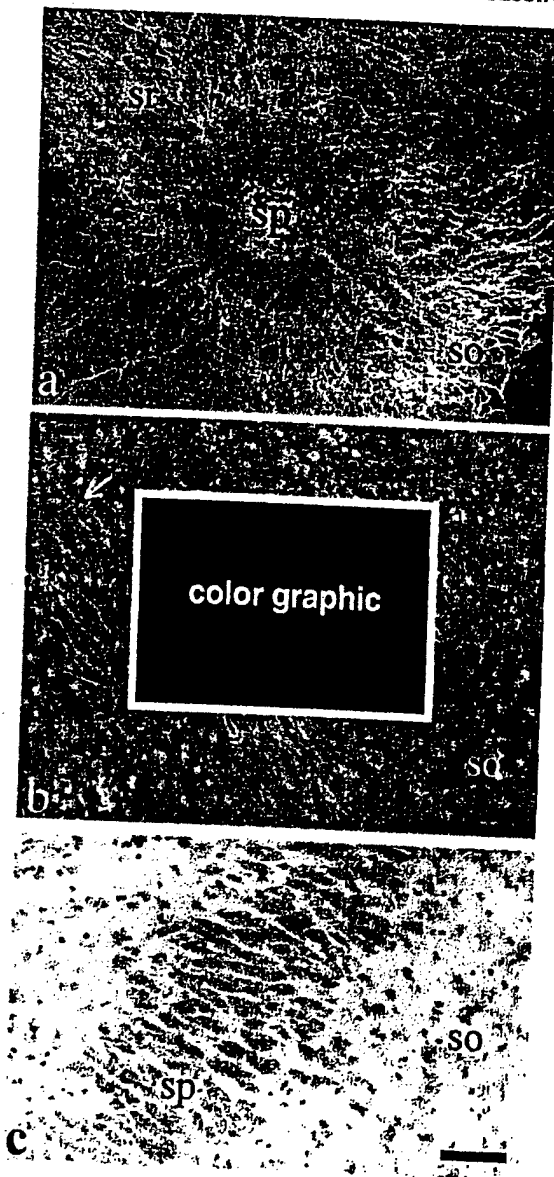
Fig. 11. Lysosomal disturbance alters the transport of endogenous tau. Immunostaining of hippocampal slice cultures with the tau-1 antibody was compared before (a) and after (b) 4 days of lysosomal dysfunction induced with chloroquine. Light, diffuse tau staining is evident in basal and apical dendritic fields of the control slice. In contrast, tau is concentrated in proximal apical dendrites in the chloroquine-treated culture. Abbreviations: sp, stratum pyramidale; sr, stratum radiatum. View-field width: 250 μm.

Fig. 10. Lysosomal disruption leads to altered transport capacity. Horseradish peroxidase (HRP) was applied to the CA1 pyramidal zone in a control slice (a) and in a slice treated with chloroquine for 6 days (b); see the boxed area in Figure 9 as a reference point for the HRP placement. Neurons were allowed to internalize and transport the HRP for 2 h, after which the cultures were fixed and stained for peroxidase activity (viewed by dark-field microscopy). Dense arrays of apical and basal dendrites exhibit transported HRP in the control slice. Despite cellular uptake, only a few apical dendrites exhibit HRP labeling in the chloroquine-treated tissue (b, arrows). Nissl staining also was conducted with hippocampal slices treated with chloroquine for 6 days (c). Abbreviations: so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Scale bars: a, b = 70 μm; c = 80 μm.
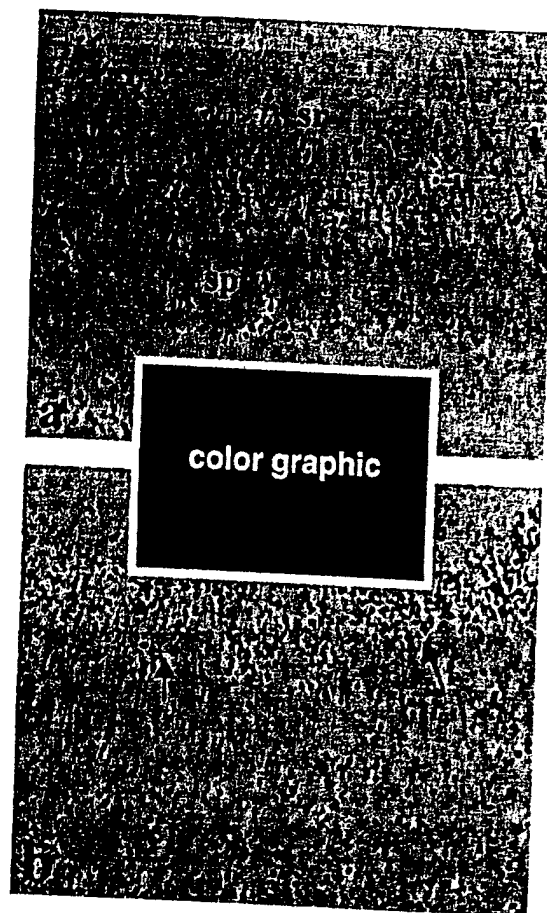
family of diseases that is associated with aggregated protein deposits (57). Together with the slice model, these data support the hypothesis that intracellular deposition promotes synaptic decay early in the pathogenesis triggered by abnormal protein processing, prior to the onset of gross cellular atrophy. Moreover, the summarized studies indicate that lysosomal perturbation is a major contributory factor in AD and perhaps other neurodegenerative disorders, as previously suggested (5, 6, 10).

Another hypothesis supported by the slice model data is that abnormal protein processing/deposition alters synaptic gene expression by disrupting microtubule integrity and related transport mechanisms necessary for synapse maintenance. For over 24 h, chloroquine had no influence on proteins directly or indirectly involved in synaptic function (12) or transport. As material gradually deposited over several days of chloroquine treatment, there was

a corresponding loss of microtubule structure as indicated by tubulin fragmentation and decreases in acetylated tubulin. Tubulin acetylation is a marker of stable microtubules and was found to be reduced in Alzheimer brains (60). In fact, the intracellular appearance of NFT aggregates coincided with diminished levels of the microtubule stability marker, thus raising the speculation that NFTs negatively impact transport processes. Not surprisingly, further indication of microtubule destabilization in the slice model was evidenced by the loss of transport capability. Over an extended period of lysosomal dysfunction, there was a correspondence that occurred between the gradual deposition of PHF material and gradual disruption of somatofugal transport.

The induced intracellular deposition formed a cap encompassing the area from which axons stem from neurons. While the deposited mass could act as an obstruction of transport corridors, the accumulation could merely be an indicator of material that failed to be transported in that direction. Such indications of early transport dysfunction have been found associated with neurodegenerative disease states (61, 62). If the deposited aggregates play a role in obstructing the movement of cellular components, physical obstruction cannot be the only reason for transport failure. HRP delivery along dendrites was reduced regardless of whether it was in the direction of the polar deposition. Changes in tau localization by chloroquine and specific lysosomal inhibitors further indicate transport failure in the direction opposite the polar accumulation. Other events associated with abnormal protein processing and PHF formation must contribute to transport dysfunction. The likely candidate is the loss of microtubule integrity. Irregular tau processing may cripple transport mechanisms by driving the tau–phospho-tau equilibrium to the right as phospho-tau transforms into PHFs, thus lowering the availability of normal tau for stabilizing microtubules. Alternatively, the abnormal processing may disrupt tau's ability to promote microtubule assembly independent of PHF formation. Note that about 20% of slices displayed transport impairment after 3 days of lysosomal dysfunction, a time at which the cultures exhibited decreased acetylated tubulin content but only trace levels of polar deposition. Soluble forms of defective human tau have been shown to cause neurodegeneration without forming tangle aggregates in fruit flies (63). Transport mechanisms are necessary for cell viability, and transport dysfunction indeed has been implicated in a number of neurodegenerative conditions (60–62, 64). In addition to the link between tau changes and transport failure, a recent study suggests that amyloidogenic processing would stall kinesin-mediated axonal transport (65). Thus, multiple avenues of abnormal processing have the potential to disrupt transport systems that are vital for synapse function.

Following lysosomal dysfunction, protein deposits first accumulated in the surrounding area of the axon hillock. The initial deposition may indicate that axons are affected by transport failure before dendrites, as similarly indicated with regards to accumulations of huntingtin aggregates, Lewy body-like inclusions, and lysosomes (61, 62). The early deposits may serve to disrupt transport processes into axons, or simply reflect the result of axonal transport dysfunction. In either case, the localized accumulation provides an explanation for the faster decline of presynaptic proteins as compared to postsynaptic markers. The apparent presynaptic vulnerability is evident at the protein level (12) as well as the message level. Reduced gene expression likely occurs as transport disruption depletes the resources of synaptic proteins, leading to the generation of signals that regulate transcription. Alternatively, somatic buildup of a protein due to transport blockage could produce a negative feedback response, thereby ceasing the expression of that particular protein. It is of interest that the translation of tau appears to occur at the proximal axon (66); thus, abnormal processing/aggregation of newly synthesized tau could promote the early deposition and axon vulnerability reported here. Lysosomal dysfunction was previously implicated in the reduction of axonal excitability (12), perhaps due to altered tau chemistries or by the depletion of transported components.

The present report shows that altered transcription affects synaptophysin levels before GluR1 expression. A decline in presynaptic elements may be responsible for the reduced expression of postsynaptic receptors. The slice model reproduces the kinds of reductions in glutamatergic receptors found in Alzheimer brains (67, 68). Following disruption of microtubule-based transport mechanisms, rapid turnover of presynaptic proteins could promote early transcriptional regulation that translates into delayed postsynaptic decline. Disrupted delivery of transmitter release machinery may impact postsynaptic structures by diminishing synapse maintenance signals and thereby initiating secondary pathology (12, 69, 71). The inter-dependencies between pre- and postsynaptic elements warrant the possibility that the receptor loss is a consequence of reduced neurotransmission and/or deterioration of axons and axon terminals.

Overall, the results indicate that lysosomal dysfunction precipitates a series of pathogenic changes leading ultimately to transport failure, and this has a negative effect on synapses in several ways. The reported connection between protein deposition, microtubule destabilization, and transport disturbances further suggests that episodes of lysosomal disturbance play a part in the pathogenesis of age-related neurodegeneration, especially synaptic pathology.

ACKNOWLEDGMENTS

REFERENCES

1. Clark CM, Trojanowski JQ. Neurodegenerative dementias: Clinical features and pathological mechanisms. New York: McGraw-Hill, 2000

2. DiFiglia M, Sapp E, Chase KO, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 1997;277:1990–93

3. Sapp E, Schwarz C, Chase K, et al. Huntingtin localization in brains of normal and Huntington's disease patients. Ann Neurol 1997;42: 604–12

4. Fahn S, Przedborski S. Parkinsonism. In: Rowland LP, ed. Merritt's textbook of neurology. Philadelphia: Williams and Wilkins, 2000: 679–93

5. Cataldo AM, Hamilton DJ, Barnett JL, Paskevich PA, Nixon RA. Properties of the endosomal-lysosomal system in the human central nervous system: Disturbance mark most neurons in populations at risk to degenerate in Alzheimer's disease. J Neurosci 1996;16:186–99

6. Kegel KB, Kim M, Sapp E, et al. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. J Neurosci 2000;20:7268–78

7. Stefanis L, Larsen KE, Rideout HJ, Sulzer D, Greene LA. Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alteration of the ubiquitin-dependent degradation system, loss of dopamine release and autophagic cell death. J Neurosci 2001;21:9549–60

8. Benowitz LI, Rodriguez W, Paskevich P, et al. The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer disease subjects. Exp Neurol 1989;106:237–50

9. Cataldo AM, Nixon RA. Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain. Proc Natl Acad Sci USA 1990;87:3861–65

10. Nixon RA, Cataldo AM, Paskevich PA, et al. The lysosomal system in neurons. Involvement at multiple stages of Alzheimer's disease pathogenesis. Ann NY Acad Sci 1992;674:65–88

11. Haass C, Koo EH, Mellon A, Hung AY, Selkoe DJ. Targeting of cell-surface beta-amyloid precursor protein to lysosomes: Alternative processing into amyloid-bearing fragments. Nature 1992;357: 500–503

12. Bahr BA, Abai B, Gall CM, et al. Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 1994;129:81–94

13. Bahr BA, Hoffman KB, Yang AJ, et al. Amyloid beta protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J Comp Neurol 1998;397:139–47

14. Yang AJ, Knauer M, Burdick DA, Glabe C. Intracellular A beta 1–42 aggregates stimulate the accumulation of stable, insoluble amyloidogenic fragments of the amyloid precursor protein in transfected cells. J Biol Chem 1995;270:14786–92

15. Chen F, Yang DS, Petanceska S, et al. Carboxyl-terminal fragments of Alzheimer beta-amyloid precursor protein accumulate in restricted and unpredicted intracellular compartments in presenilin 1-deficient cells. J Biol Chem 2000;275:36794–802

16. Hajimohammadreza I, Anderson VER, Cavanagh JB, et al. β-Amyloid precursor protein fragments and lysosomal dense bodies are found in rat brain neurons after ventricular infusion of leupeptin. Brain Res 1994;640:25–32

17. Hayashi Y, Kashiwagi K, Yoshikawa K. Protease inhibitors generate cytotoxic fragments from Alzheimer amyloid protein precursor

18. Siman R, Mistretta S, Durkin JT, et al. Processing of the beta-amyloid precursor. Multiple proteases generate and degrade potentially amyloidogenic fragments. J Biol Chem 1993;268:16602–9

19. Ivy GO. Protease inhibition causes some manifestations of aging and Alzheimer's disease in rodent and primate brain. Ann NY Acad Sci 1992;674:89–102

20. Cavanagh JB, Nolan CC, Seville MP, Anderson VE, Leigh PN. Routes of excretion of neuronal lysosomal dense bodies after ventricular infusion of leupeptin in the rat: A study using ubiquitin and PGP 9.5 immunocytochemistry. J Neurocytol 1993;22:779–91

21. Bahr BA. Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 1995;42:294–305

22. Takauchi S, Miyoshi K. Cytoskeletal changes in rat cortical neurons induced by long-term intraventricular infusion of leupeptin. Acta Neuropathol 1995;89:8–16

23. Bi X, Zhou J, Lynch G. Lysosomal protease inhibitors induce meganeurites and tangle-like structures in entorhinohippocampal regions vulnerable to Alzheimer's disease. Exp Neurol 1999;158: 312–27

24. Wisniewski HM, Narang HK, Terry RD. Neurofibrillary tangles of paired helical filaments. J Neurol Sci 1976;27:173–81

25. Kosik KS, Joachim CL, Selkoe DJ. Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci USA 1986;83:4044–48

26. Wood JG, Mirra SS, Pollock NJ, Binder LI. Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau. Proc Natl Acad Sci USA 1986; 83:4040–43

27. Lee VM, Balin BJ, Otvos L Jr, Trojanowski JQ. A68: A major subunit of paired helical filaments and derivatized forms of normal tau. Science 1991;251:675–78

28. Bahr BA, Vicente JS. Age-related phosphorylation and fragmentation events influence the distribution profiles of distinct tau isoforms in mouse brain. J Neuropathol Exp Neurol 1998;57:111–21

29. Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 1992;42:631–39

30. Haroutunian V, Purohit DP, Perl DP, et al. Neurofibrillary tangles in nondemented elderly subjects and mild Alzheimer disease. Arch Neurol 1999;56:713–18

31. Callahan LM, Coleman PD. Neurons bearing neurofibrillary tangles are responsible for selected synaptic deficits in Alzheimer's disease. Neurobiol Aging 1995;16:311–14

32. Eastwood SL, Burnet PW, McDonald B, Clinton J, Harrison PJ. Synaptophysin gene expression in human brain: A quantitative in situ hybridization and immunocytochemical study. Neurosci 1994; 59:881–92

33. Masliah E, Terry RD, DeTeresa RM, Hansen LA. Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. Neurosci Lett 1989;103:234–39

34. Lassmann H, Fisher P, Jellinger K. Synaptic pathology of Alzheimer's disease. Ann NY Acad Sci 1993;24:59–64

35. Samuel W, Masliah E, Hill LR, Butters N, Terry R. Hippocampal connectivity and Alzheimer's dementia: Effects of synapse loss and tangle frequency in a two-component model. Neurology 1994;44: 2081–88

36. Caporaso GL, Gandy SE, Buxbaum JD, Greengard P. Chloroquine inhibits intracellular degradation but not secretion of Alzheimer ☐/ A4 amyloid precursor protein. Proc Natl Acad Sci USA 1992;89: 2252–56

37. Golde TE, Estus S, Younkin LH, Selkoe DJ, Younkin SG. Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. Science 1992;255:728–30

38. Wibo M, Poole B. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B1. J Cell Biol 1974;63: 430–40

39. Bahr BA, Hoffman KB, Kessler M, et al. Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (GR53) in brain tissue. Neurosci 1996;74:707–21

40. Guttmann RP, Baker DL, Seifert KM, et al. Specific proteolysis of the NR2 subunit at multiple sites by calpain. J Neurochem 2001; 78:1083–93

41. Bahr BA, Lam N, Lynch G. Changes in the concentrations of tau and other structural proteins in the brains of aged mice. Neurosci Lett 1994;175:49–52

42. Delacourte A, Flament S, Dibe EM, et al. Pathological proteins Tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease. Demonstration with a panel of antibodies against Tau proteins. Acta Neuropathol (Berl) 1990;80:111–17

43. Goedert M, Spillantini MG, Cairns NJ, Crowther RA. Tau proteins of Alzheimer paired helical filaments: Abnormal phosphorylation of all six brain isoforms. Neuron 1992;8:159–68

44. Black MM, Baas PW, Humphries S. Dynamics of alpha-tubulin deacetylation in intact neurons. J Neurosci 1989;9:358–68

45. Heinonen O, Soininen H, Sorvari H, et al. Loss of synaptophysin-like immunoreactivity in the hippocampal formation is an early phenomenon in Alzheimer's disease. Neurosci 1995;54:375–84

46. Callahan LM, Vaules WA, Coleman PD. Quantitative decrease in synaptophysin message expression and increase in cathepsin D message expression in Alzheimer disease neurons containing neurofibrillary tangles. J Neuropathol Exp Neurol 1999;58:275–87

47. Pasinetti GM. Use of cDNA microarray in the search for molecular markers involved in the onset of Alzheimer's disease dementia. J Neurosci Res 2001;65:471–76

48. Perdahl E, Adolfsson R, Alafuzoff I, et al. Synapsin I (Protein I) in different brain regions in senile dementia of Alzheimer type and in multiinfarct dementia. J Neural Transm 1984;60:133–41

49. Honer WG, Dickson DW, Gleeson J, Davies P. Regional synaptic pathology in Alzheimer's disease. Neurobiol Aging 1992;13:375–82

50. Lassmann H, Weiler P, Fischer P, et al. Synaptic pathology in Alzheimer's disease: Immunological data for markers of synaptic and large dense-core vesicles. Neurosci 1992;46:1–8

51. Terry RD, Masliah E, Salmon DP, et al. Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. Ann Neurol 1991;30:572–80

52. Blennow K, Bogdanovic N, Alafuzoff I, Ekman R, Davidsson P. Synaptic pathology in Alzheimer's disease: Relation to severity of dementia, but not to senile plaques, neurofibrillary tangles, or the ApoE4 allele. J Neural Transm 1996;103:603–18

53. Scheff SW, Sparks DL, Price DA. Quantitative assessment of synaptic density in the outer molecular layer of the hippocampal dentate gyrus in Alzheimer's disease. Dementia 1996;7:226–32

54. Ikeda K, Akiyama H, Arai T, et al. Alz-50/Gallyas-positive lysosome-like intraneuronal granules in Alzheimer's disease and control brains. Neurosci Lett 1998;258:113–16

55. Oyama F, Murakami N, Ihara Y. Chloroquine myopathy suggests that tau is degraded in lysosomes: Implication for the formation of paired helical filaments in Alzheimer's disease. Neurosci Res 1998; 31:1–8

56. Auer IA, Schmidt ML, Lee VM, et al. Paired helical filament tau (PHFtau) in Niemann-Pick type C disease is similar to PHF-tau in Alzheimer's disease. Acta Neuropathol (Berl) 1995;90:547–51

57. Laszlo L, Lowe J, Self T, et al. Lysosomes as key organelles in the pathogenesis of prion encephalopathies. J Pathol 1992;166:333–41

58. Ohkuma S, Poole B. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci USA 1978;75:3327–31

59. Scornik OA. Effects of inhibitors of protein degradation on the rate of protein synthesis in Chinese hamster ovary cells. J Cell Physiol 1984;121:257–62

60. Hempen B, Brion JP. Reduction of acetylated α-tubulin immuno-reactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease. J Neuropathol Exp Neurol 1996;55:964–72

61. Sasaki S, Iwata M. Impairment of fast axonal transport in the proximal axons of anterior horn neurons in amyotrophic lateral sclerosis. Neurology 1996;47:535–40

62. Li H, Li S-H, Yu Z-X, Shelbourne P, Li X-J. Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. J Neurosci 2001;21:8473–81

63. Wittmann CW, Wszolek MF, Shulman JM, et al. Tauopathy in Drosophila: Neurodegeneration without neurofibrillary tangles. Science 2001;293:711–14

64. Griffin JW, Watson DF. Axonal transport in neurological disease. Ann Neurol 1988;23:3–13

65. Kamal A, Almenar-Queralt A, LeBlanc JF, Roberts EA, Goldstein LS. Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature 2001;414:643–48

66. Mandelkow EM, Biernat J, Drewes G, et al. Tau domains, phosphorylation, and interactions with microtubules. Neurobiol Aging 1995;16:355–62

67. Wakabayashi K, Narisawa-Saito M, Iwakura Y, et al. Phenotypic down-regulation of glutamate receptor subunit GluR1 in Alzheimer's disease. Neurobiol Aging 1999;20:287–95

68. Hynd MR, Scott HL, Dodd PR. Glutamate (NMDA) receptor NR1 subunit mRNA expression in Alzheimer's disease. J Neurochem 2001;78:175–82

69. Bahr BA, Bendiske J. The neuropathogenic contribution of lysosomal dysfunction. J Neurochem 2002; in press.

70. Caba E, Brown QB, Kawasaki B, Bahr BA. Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. J Neurosci Res 2002;67:787–94

71. Bahr BA, Bendiske J, Brown QB, et al. Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol 2002;174:37–47

Figure 1

Figure 2

lysosomal disruption, 6 d

| − | + | − | + |

←BDP$_N$
←NR1
←GluR1

← actin

Figure 3

a

days: 0 3 6
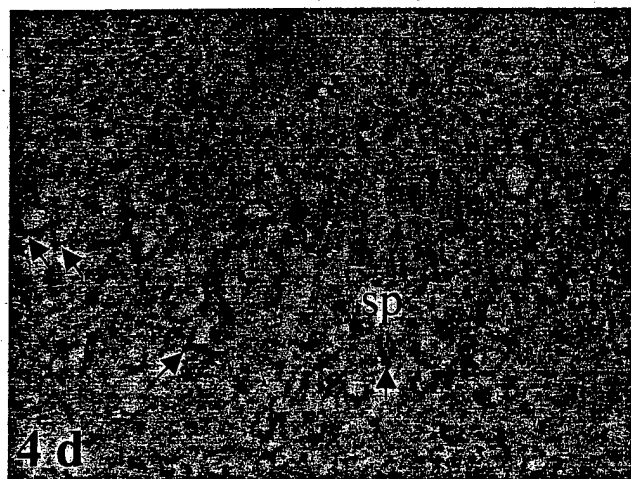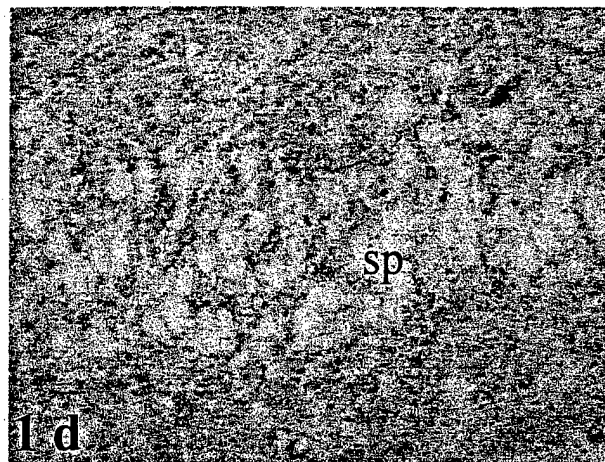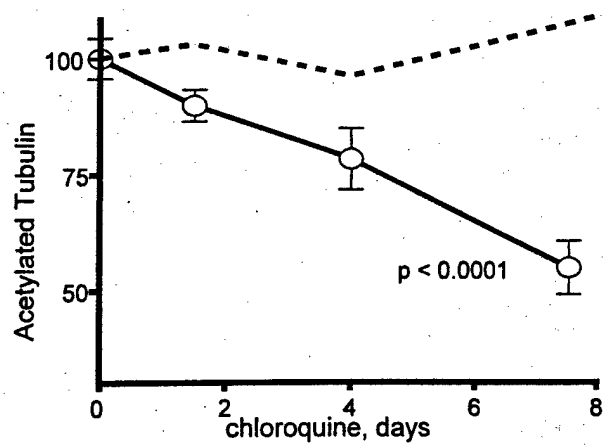

synaptophysin

GluR1

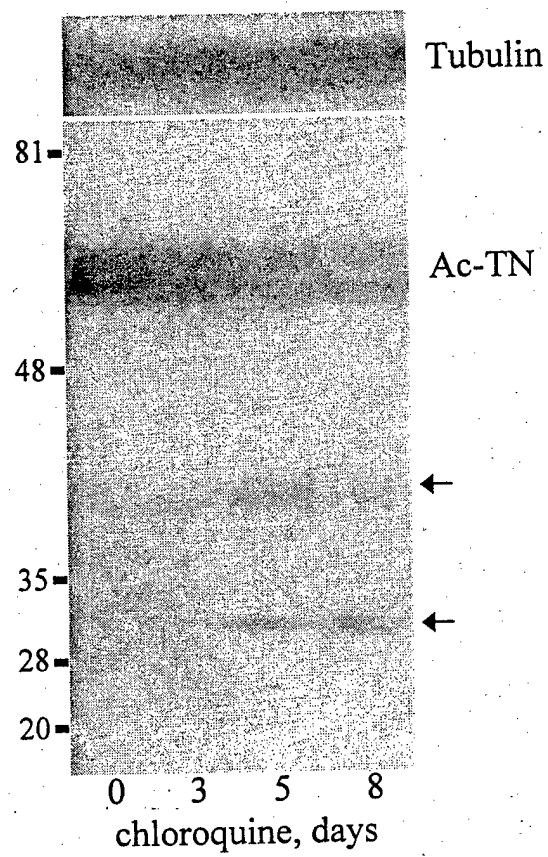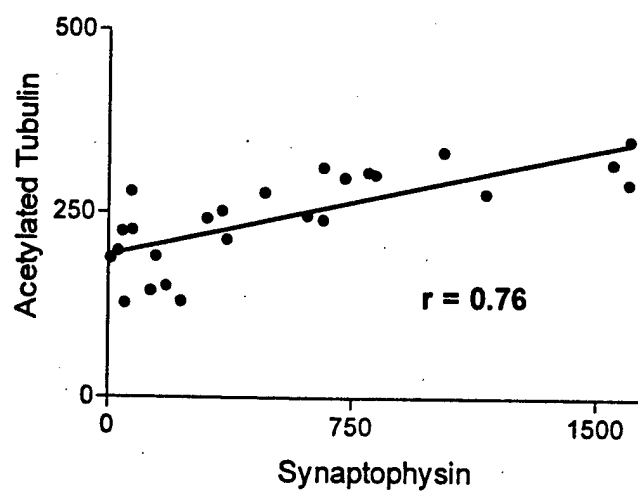actin

b

days: 0 3 6 9

Syn

GluR1

HPRT
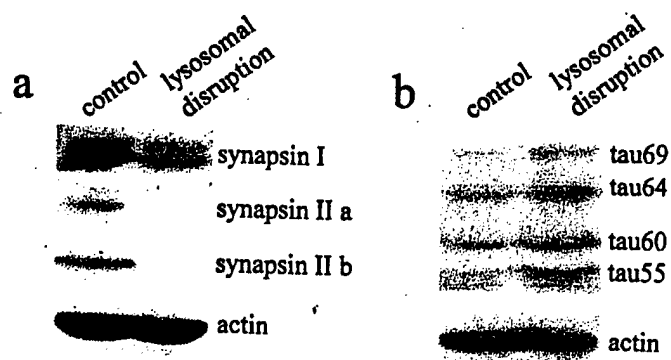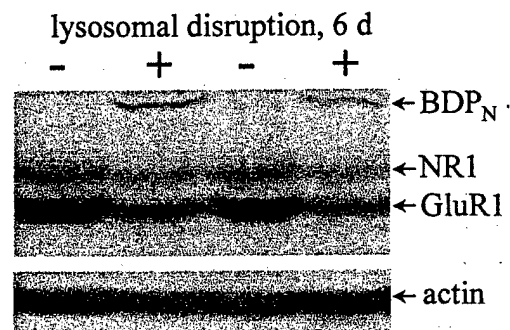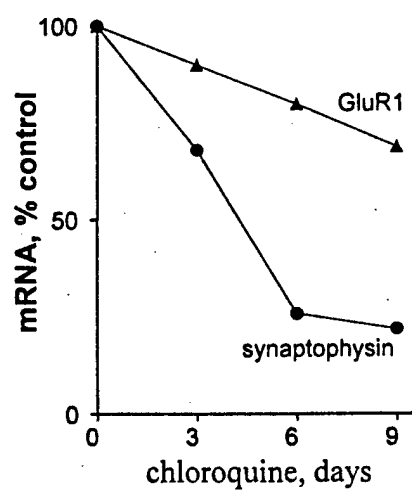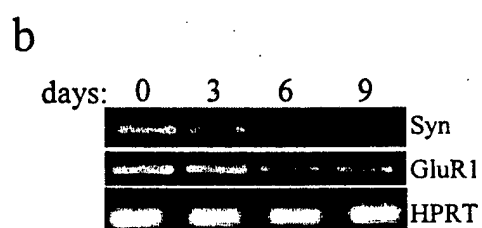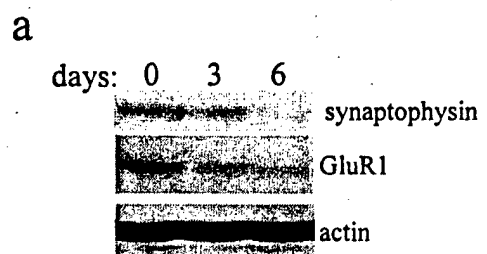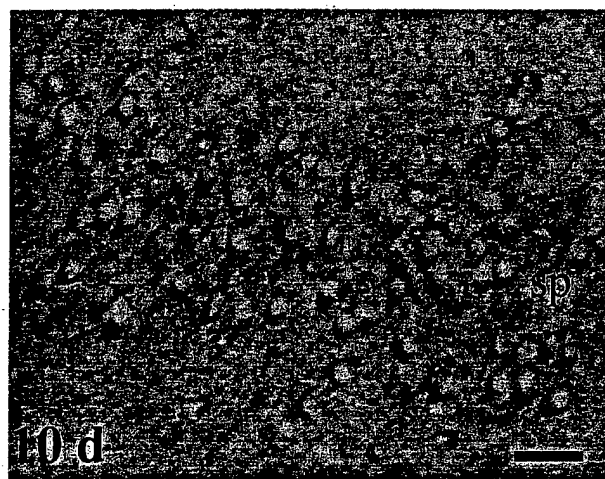
Figure 4

Figure 5
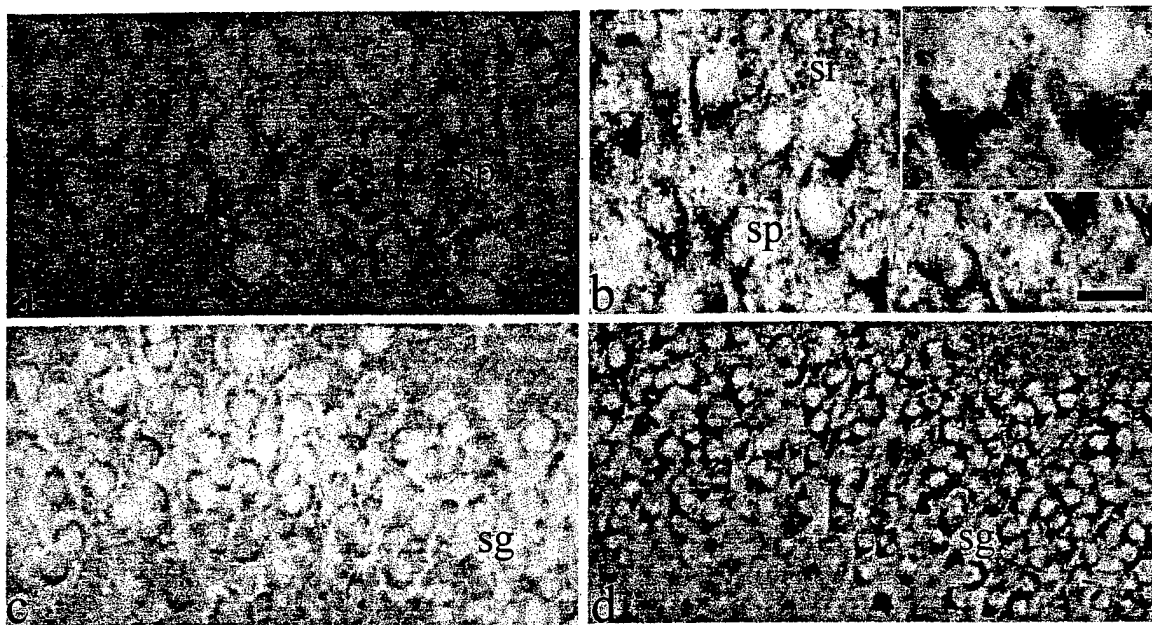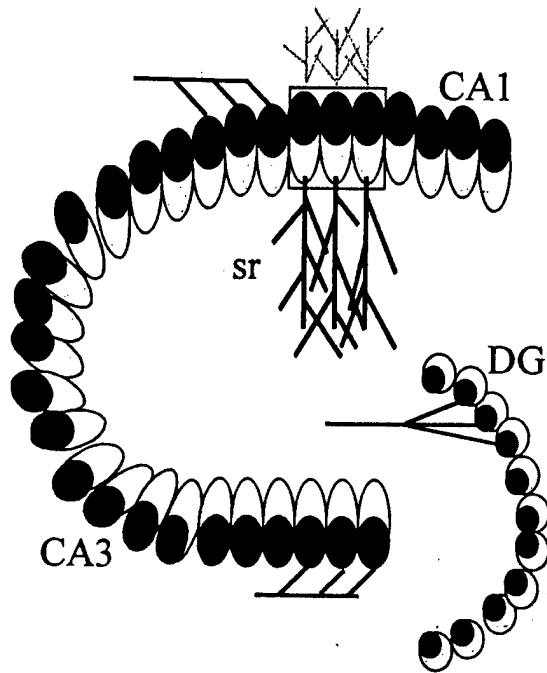
Figure 6

Figure 7

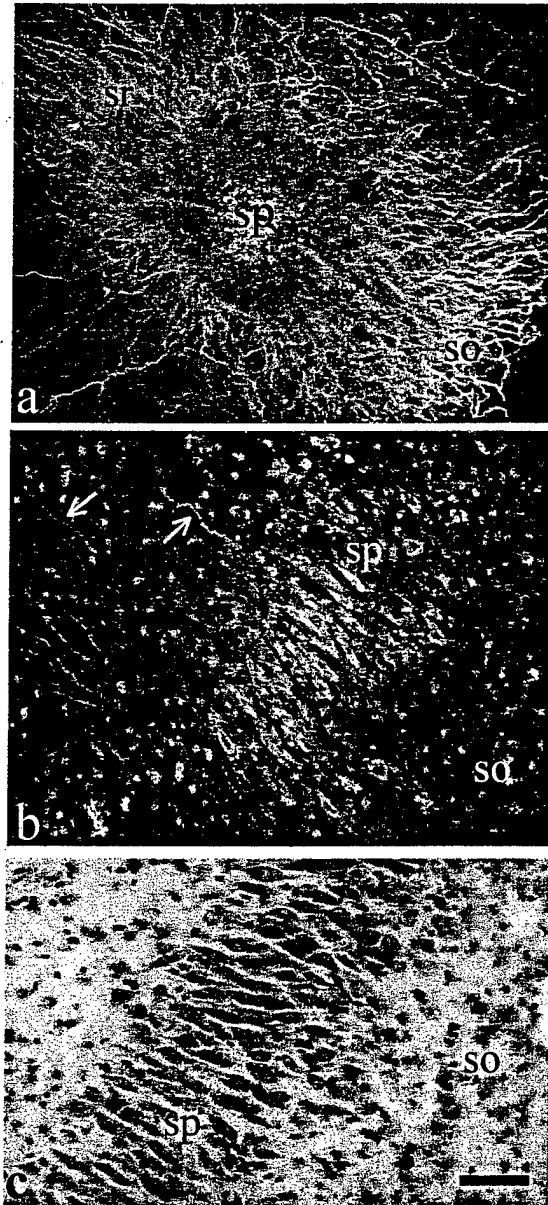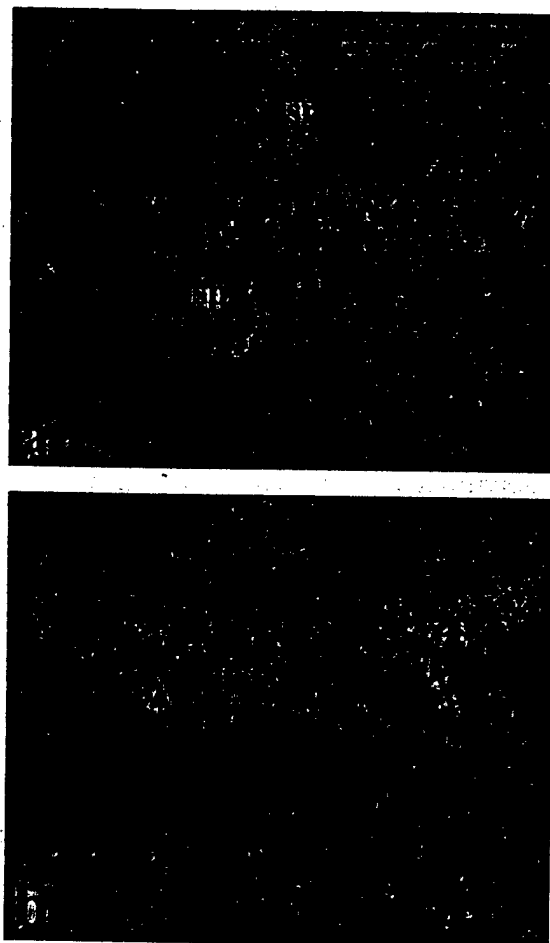Figure 8

Figure 9

Figure 10

Figure 11

Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age

Joycelyne S.Vicente[1], Subramani Munirathinam[2], and Ben A. Bahr[2]

[1]Center for the Neurobiology of Learning and Memory, University of California, Irvine, California, USA
[2]Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut, USA

The cytoskeletal protein spectrin is targeted by the calcium-dependent protease calpain in different types of pathologies, during which activation of the protease occurs in the most vulnerable neuronal populations. In order to identify areas vulnerable to neurodegeneration during initial stages of brain aging, tissue from 3- and 12-month mice were assessed for the calpain-mediated spectrin breakdown product BDP_N. Neocortical and hippocampal samples from the middle-aged mice contained two- to three-fold more BDP_N than those samples prepared from the young group of mice. In contrast, no age-related change in spectrin fragmentation was evident in olfactory bulb, thalamic-rich, or hindbrain homogenates. Immunocytochemical analyses revealed focal labeling of BDP_N along the dendrites and the cell body membrane of cortical neurons in 12- but not 3-month mice. CA1 pyramidal neurons of the middle-aged mice exhibited similar immunostaining, while the other hippocampal subfields did not. These observations provide evidence that calpain activation contributes to the aging processes that potentiate neuronal vulnerability.

contact: Ben A. Bahr, Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269
e-mail: Bahr@uconn.edu

1. Introduction

Spectrin is an important cytoskeletal component for the structural composition of neurons. The spectrin network is susceptible to proteolytic damage during a variety of pathogenic processes (see ref. 44 for a recent review) as well as during normal aging [3,5,34]. The proteolysis of spectrin is largely mediated by calpain, a calcium-activated thiol protease found widely distributed in the brain [16,33]. Calpain activation follows the change in intracellular calcium in vulnerable neurons resulting from neurodegenerative insults including brain trauma [35,37], ischemic events in neonatal [10,11] and adult animals [36,39], and exposure to neurotoxins [4,41,42]. Earlier studies have also suggested that calpain-mediated proteolysis occurs in neurons vulnerable to the aging process [3,5]. Thus, periodic episodes of pathogenic calpain activation may render select regions susceptible to neurodegeneration in the aged brain.

The present study tested whether calpain-mediated spectrin breakdown product is produced at early stages of brain aging, and whether this marker of neurodegeneration identifies neurons that are particularly vulnerable at later stages. Antibodies to the calpain cleavage site in the spectrin molecule were used to selectively measure the amino-terminal breakdown product (BDP_N). Brain regions known to exhibit neuronal loss with aging were found to possess greatly increased levels of BDP_N in middle-aged mice.

2. Methods

Three-, 12-, and 18-month-old male mice from the hybrid stock of B6D271 (C57BL/6 x DBA/2) were obtained from the National Institute on Aging and observed for health for a week. For the preparation of immunoblot samples, animals were sacrificed by metofane anesthesia and decapitation. Each brain was rapidly cooled, removed from the skull, and immediately dissected in ice-cold homogenization buffer containing 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, and the protease inhibitors antipain (10 µg/ml), aprotinin, leupeptin, pepstatin A (each at 2 µg/ml), and 0.3 mM phenylmethylsulfonyl fluoride. Brain stem and cerebellum were separated from the endbrain

and combined together. After removal of the olfactory bulbs, the loosely attached core of the caudal two-thirds of the endbrain containing primarily thalamus and hypothalamus was collected. The neocortical tissue remained after dissecting away the hippocampus from the cortical shell. The regions were quickly homogenized in fresh homogenization buffer. Homogenate samples denatured in 2.5% sodium dodecyl sulfate and 3% β-mercaptoethanol were then subjected to electrophoresis on polyacrylamide gradient gels (3-17%) and transferred to 0.2-μm nitrocellulose as previously described (see ref. 6).

Affinity-purified antibodies to latter residues of the aminoterminal spectrin fragment produced by calpain, anti-BDP_N [4], were used to stain immunoblots. Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and color development used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Color development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Linearity tests were conducted with 3- and 12-month mice samples as well as with 3-month rat forebrain membranes that were incubated with 2 mM $CaCl_2$ at 37°C for 60 min in order to be positive for calpain-mediated proteolysis. Image analysis was used to obtain BDP_N optical density and image area measures, and the typical protein vs. immunoreactivity correlation was 0.99.

For immunocytochemistry studies, animals under deep pentobarbital anesthesia were perfused intracardially with 0.1 M phosphate buffer (PB; pH 7.4), then with PB containing 4% paraformaldehyde. The fixed brains were cryoprotected in cold PB containing 20% sucrose overnight. Using the avidin-biotin-peroxidase technique, microtome sections of 30 μm were stained with affinity-purified anti-BDP_N antibodies.

3. Results

Affinity-purified antibodies raised against the calpain recognition sequence were used to label the 150-kDa spectrin breakdown product BDP_N in tissue homogenates prepared from 3- and 12-month mice. Samples from olfactory bulb (O), neocortex (N), hippocampus (H), thalamic-rich tissue (T), and cerebellar-brain stem material (C) are shown in the anti-BDP_N immunoblots of Figure 1. Different amounts of rat brain membranes exhibiting calpain-mediated proteolysis were included on the immunoblots as positive controls (lanes 1 and 2). Relative immunoreactivity levels of BDP_N were determined by integrated density measurements from an image analysis system. The spectrin breakdown marker exhibited a pronounced difference among brain regions isolated from the middle-aged mice (p<0.001, nonparametric ordinary ANOVA; Kruskal-Wallis statistic = 19.5), while there was no significant difference in the BDP_N levels across the 3-month samples (p=0.29). The olfactory bulb contained particularly low levels of BDP_N in the 12-month samples. Note that the anti-BDP_N antibodies specifically recognized the calpain-mediated fragment as previously shown [4], and did not label the parent spectrin which was verified to have migrated to the 240-kDa position on the immunoblots (labeled S).

Age-related increases in BDP_N were evident in neocortical and hippocampal tissue samples, but not in the tissue of the olfactory bulb, thalamus, or hindbrain (Fig. 1, lower panel). In neocortex, the eight mice 12 months of age contained three-fold more spectrin breakdown than that found in samples from the nine young mice (p=0.01, nonparametric test). Hippocampal tissue exhibited a two-fold increase in the calpain-mediated spectrin fragment between 3 and 12 months of age (p<0.01). The increases in BDP_N at middle age were confirmed by normalizing the individual immunoreactivity values to a standard curve (r=0.99; p<0.0001) generated from blot samples containing increasing amounts of rat brain membranes positive for spectrin fragmentation. The normalized data exhibited the same fold increases between the 3- and 12-month samples as determined by the integrated density measures. In addition, similar results with regards to magnitude and regional selectivity were found in samples from four mice 18 months of age.

In immunocytochemistry studies, only trace, diffuse labeling of BDP_N was evident in 3-month brain tissue, most likely as a result of regular spectrin turnover events. However, in

12-month mice, distinct tissue staining was evident in the two regions identified from BDP_N immunoblots as vulnerable to calpain-mediated damage. In the cortex, neurons in layer V were immunopositive for the spectrin fragment found concentrated in focal zones along dendrites and the circumference of neuronal cell bodies. This was seen in mice 12 months of age (Fig. 2b) but not in 3-month animals (Fig. 2a). Similar age-related immunostaining was seen in the region of the entorhinal cortex (data not shown). In the hippocampus, the middle-aged mice had distinctive punctate staining of BDP_N in the CA1 pyramidal layer and neighboring dendritic fields, and such staining was not evident in the young animals (Fig. 3a vs. 3b). The age-related immunoreactivity was most obvious in the CA1a region and extended into the subiculum. In field CA3 and the dentate gyrus, only low levels of diffuse BDP_N staining were found in 3- (Fig. 3c) and 12-month mice (Fig. 3d). When the primary antibody was removed from the immunocytochemistry procedure, no labeling occurred in any brain region.

4. Discussion

The present findings indicate that proteolytic damage occurs in a subset of neurons early during the brain aging process. The spectrin fragment BDP_N produced by calpain is a reliable marker of neurotoxicity and several disease states, and was shown here to accumulate in select brain regions of middle-aged mice. While no apparent change in the parent spectrin was found between 3 and 12 months of age, BDP_N concentrations increased two- to three-fold in the cortex and hippocampus. The spectrin breakdown event thereby provides an extremely sensitive marker of age-related changes, occurring early when total spectrin and thus cellular integrity are not significantly altered. The middle-age brain regions identified as being affected by calpain activity are particularly vulnerable in the aged human brain [26,28]. The same brain regions also are vulnerable to calpain-mediated damage early after an excitotoxic insult [36,39]. Therefore, synaptic excitability at middle age may lead to localized changes in cellular calcium to produce the synapse-like focal zones of calpain activation shown here. Calcium regulation is especially compromised in regions

that exhibit damage in the aged brain [14,20,45], further indicating that aging potentiates vulnerability to calcium-dependent neurodegenerative events. These findings and the present data also add support to the idea that neuronal vulnerability to excitotoxic damage is increased by age-related processes [1,13,23,27,46].

The selective accumulation of BDP_N corresponds with the regional selectivity exhibited by age-related synaptic deterioration [2] and with changes in cytoskeletal and microtubule components that also arise at middle age and persist late into the lifespan of mice [1,5,6]. Besides proteolytic damage to the structural nature of neurons, calpain activation may also induce damage to lysosomal membranes. For example, the persistent cytoskeletal damage initiated at middle age correlates with the progressive destabilization of neuronal lysosomes that starts early in the aging rat brain [22, 24,30, 31], in the aging human brain [8], and in age-related disorders [12,15]. The activated form of calpain was localized to disrupted lysosomal membranes of CA1 neurons prior to cell death in the ischemic primate brain (see ref. 47). In addition, lysosomal disruption and resultant leakage of contents were evident only in the ischemia-vulnerable CA1 neurons [48], the same subfield found to exhibit the earliest and most pronounced level of calpain activation (see ref. 44). Thus, the calpain responses found in Alzheimer's disease and Parkinson's disease [17,25,29,32,40] may explain why lysosomal dysfunction and upregulation of lysosomal hydrolases have been linked to age-related types of pathogenesis [1,7,15,18, 19,21,43,49].

The constant production of spectrin fragments throughout the span of aging could weaken protein degradation pathways in lysosomes, possibly inducing feedback mechanisms to upregulate hydrolase levels. Persistent calpain activation events also would cause proteolytic degradation of the endogenous inhibitor calpastatin [10,38], thereby promoting unregulated proteolysis and further lysosomal strain. Interestingly, vulnerable brain regions that express calpain proteolysis at middle age also exhibit activation of compensatory systems in response to lysosomal stress. Specifically, the lysosomal hydrolase cathepsin D increased in

hippocampal field CA1 but not CA3 in 12-month animals [9], the same regional selectivity shown here for calpain-mediated BDP$_N$. Together with the earlier discussion, these findings suggest that calpain plays a role in the abnormal protein processing involved in age-related disorders, and that increases in lysosomal hydrolases with aging and ischemia occur, at least in part, to compensate for calpain-mediated cellular changes.

Acknowledgements

References

[1] Bahr BA, Abai B, Gall CM, Vanderklish PW, Hoffman KB, Lynch G. Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 1994; 129:81-94.

[2] Bahr BA, Godshall AC, Hall RA, Lynch G. Mouse telencephalon exhibits an age-related decrease in glutamate (AMPA) receptors but no change in nerve terminal markers. Brain Res 1992; 589:320-326.

[3] Bahr BA, Lam N, Lynch G. Changes in the concentrations of tau and other structural proteins in the brains of aged mice. Neurosci Lett 1994; 175:49-52.

[4] Bahr BA, Tiriveedhi S, Park GY, Lynch G. Induction of calpain-mediated spectrin fragments by pathogenic treatment in long-term hippocampal slices. J Pharmacol Exp Ther 1995; 273:902-908.

[5] Bahr BA, Vanderklish, PW, Ha LT, Tin MT, Lynch, G. Spectrin breakdown products increase with age in telencephalon of mouse brain. Neurosci Lett 1991; 131:237-240.

[6] Bahr BA, Vicente JS. Age-related phosphorylation and fragmentation events influence the distribution profiles of distinct tau isoforms in mouse brain. J Neuropathol Exp Neurol 1998; 57:111-121.

[7] Benowitz LI, Rodriguez W, Paskevich P, Mufson EJ, Schenk D, Neve RL. The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer disease subjects. Exp Neurol 1990; 106: 237-250.

[8] Bernstein HG, Kirschke H, Wiederanders B, Schmidt D, Rinne A. Antigenic expression of cathepsin B in aged human brain. Brain Res Bull 1990; 24:17-19.

[9] Bi Y, Yong AP, Zhou J, Gall CM, Lynch G. Regionally selective changes in brain lysosomes occur in the transition from young adulthood to middle age in rats. Neurosci 2000; 97:395-404.

[10] Blomgren K, Hallin U, Andersson AL, Puka-Sundvall M, Bahr BA, McRae A, Saido TC, Kawashima S, Hagberg H. Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. J Biol Chem 1999; 274:14046-14052.

[11] Blomgren K, McRae A, Elmered A, Bona E, Kawashima S, Saido TC, Ono T, Hagberg H. The calpain proteolytic system in neonatal hypoxic-ischemia. Ann NY Acad Sci 1997; 825:104-119.

[12] Bowen DM, Smith CB, Davison AN. Molecular changes in senile dementia. Brain 1973; 96:849-856.

[13] Brouillet E, Jenkins BG, Hyman BT, Ferrante RJ, Kowall NW, Srivasatava R, Roy DS, Rosen BR, Beal MF. Age-dependent vulnerability of the striatum to the mitochondrial toxin 3-nitropropionic acid. J Neurochem 1993; 60:356-359.

[14] Campbell LW, Hao SY, Thibaualt O, Blalock EM, Landfield PW. Aging changes in voltage-gated calcium currents in hippocampal CA1 neurons. J Neurosci 1996; 16:6286-6295.

[15] Cataldo AM, Paskevich PA, Kominami E, Nixon RA. Lysosomal hydrolases of different classes are abnormally distributed in brains of patient with Alzheimer disease. Proc Natl Acad Sci USA 1990; 88:10998-11002.

[16] Fuduka T, Adachi E, Kawashima S, Yoshiya I, Hashimoto PH. Immunohistochemical distribution of calcium-activated neutral proteinases and endogenous CANP inhibitor in the rabbit hippocampus. J Comp Neurol 1990; 302:100-109.

[17] Grynspan F, Griffin WR, Cataldo A, Katayama S, Nixon RA. Active site-directed antibodies identify calpin II as an early-appearing and pervasive component of

neurofibrillary pathology in Alzheimer's disease. Brain Res 1997; 763:145-158.

[18] Haass C, Koo EH, Mellon A, Hung AY, Selkoe DJ. Targeting of cell-surface β-amyloid precursor protein to lysosomes: Alternative processing into amyloid-bearing fragments. Nature (Lond) 1992; 357:500-503.

[19] Hajimohammadreza I, Ver A, Cavanagh JB, Seville MP, Nolan CC, Anderton BH, Leigh PN. β-Amyloid precursor protein fragments and lysosomal dense bodies are found in rat brain neurons after ventricular infusion of leupeptin. Brain Res 1994; 640:25-32.

[20] Herman JP, Chen KC, Booze R, Landfield PW. Up-regulation of alpha (1D) Ca2+ channel subunit mRNA expression in the hippocampus of aged F344 rats. Neurobiol Aging 1998; 19:581-587.

[21] Ivy GO. Protease inhibition causes some manifestations of aging and Alzheimer's Disease in rodent and primate brain. Ann NY Acad Sci 1992; 674:89-102.

[22] Kikugawa K, Beppu M, Kato T, Yamaki S, Kasai H. Accumulation of autofluorescent yellow lipofuscin in rat tissues estimated by sodium dodecylsulfate extraction. Mech Ageing Dev 1994;74:135-148.

[23] Koh JY, Yang LL, Cotman CW. β-Amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. Brain Res 1990; 533:315-320.

[24] Landfield P, Braun L, Pitler T, Lindsey J, Lynch G. Hippocampal aging in rats: A morphometric study of multiple variables in semithin sections. Neurobiol Aging 1981; 2:265-267.

[25] Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 2000; 405:360-364.

[26] Mann DMA. Sense and senility: The neuropathology of the aged human brain, New York: Chapman & Hall, 1997.

[27] Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 1992; 12:376-389.

[28] Morrison JH, Hof PR. Life and death of neurons in the aging brain. Science 1997; 78:412-419.

[29] Mouatt-Prigent A, Karlsson JO, Agid Y, Hirsch EC. Increased M-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: A role in nerve cell death? Neurosci 1996; 73:979-987.

[30] Nakamura Y, Takeda M, Suzki Morita K, Tada K, Hariguchi S, Nishimura R. Lysosome instability in aged rat brain. Neurosci Lett 1989; 97:215-220.

[31] Nakanishi H, Tominaga K, Amano T, Hirotsu I, Inoue T, Yamamoto K. Age-related changes in activities and localizations of cathepsins D, E, B and L in rat brain tissues. Exp Neruol 1994; 126:119-128.

[32] Nilsson E, Alafuzoff I, Blennow K, Blomgren K, Hall CM, Janson I, Karlsson I, Wallin A, Gottfries CG, Karlsson JO. Calpain and capastatin in normal and Alzheimer-degenerated human brain tissue. Neurobiol Aging 1990; 11:425-431.

[33] Perlmutter LS, Gall C, Baudry M, Lynch G. Distribution of calcium-activated protease calpain in rat brain. J Comp Neurol 1990; 296:269-276.

[34] Peterson C, Vanderklish PW, Seubert P, Cotman C, Lynch G. Increased spectrin proteolysis in fibroblasts from aged and Alzheimer donors. Neurosci Lett 1991; 121:239-243.

[35] Postmantur R, Kampfl A, Siman R, Liu J, Zhao X, Clifton GL, Hayes R. A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat. Neurosci 1997; 77:875-888.

[36] Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. J Neurosci 1994; 14:3934-3944.

[37] Saatman KE, Bozyczko-Coyne D, Marcy V, Siman R, McIntosh TK. Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat. J Neuropathol Exp Neurol 1996; 55:850-860.

[38] Saido TC, Kawashima S, Tani E, Yokota M. Up- and down-regulation of calpain inhibitor polypeptide, calpastatin, in postischemic hippocampus. Neurosci Lett 1997; 227:75-78.

[39] Saido TC, Yokota M, Nagao S, Yamaura I, Tani E, Tsuchiya T, Suzuki K, Kawashima S. Spatial resolution of fodrin proteolysis in

postischemic brain. J Biol Chem 1993; 268:25239-25243.

[40] Saito K, Elce JS, Hamos JE, Nixon RA. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: A potential molecular basis for neuronal degeneration. Proc Natl Acad Sci U.S.A. 1993; 90:2628-2632.

[41] Seubert P, Nakagawa Y, Ivy G, Vanderklish P, Baudry M, Lynch G. Intrahippocampal colchicine injection results in spectrin proteolysis. Neurosci 1989; 31:195-202.

[42] Siman R, Noszak JC. Excitatory amino acids activate calpain I and induce structural protein breakdown *in vivo*. Neuron 1988; 1:279-287.

[43] Takauchi S, Miyoshi K. Cytoskeletal changes in rat cortical neurons induced by long-term intraventricular infusion of leupeptin. Acta Neuropathol 1995; 89:8-16.

[44] Vanderklish PW, Bahr BA. The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 2000; 81:323-339.

[45] Verkhratsky A, Toescu EC. Calcium and neuronal ageing. Trends Neurosci 1998; 21:2-7.

[46] Wozniak DF, Stewart GR, Miller JP, Olney JW. Age-related sensitivity to kainate neurotoxicity. Exp Neurol 1991; 114:250-253.

[47] Yamashima, T. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 2000; 62:273-295.

[48] Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E. Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: A novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. Eur J Neurosci 1998; 10:1723-1733.

[49] Yang AJ, Chandswangbhuvana D, Margol L, Glabe CG. Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Abeta1-42 pathogenesis. J Neurosci Res 1998; 52:691-698.
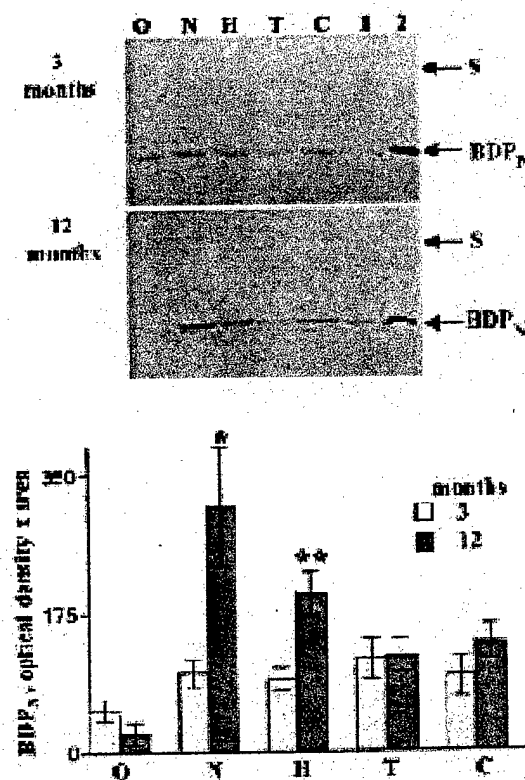
6

Figure 1. Increase in calpain-mediated proteolysis occurs in select brain regions of middle-aged mice. Equal protein aliquots (100 µg) of homogenized tissue samples from mice at 3 and 12 months of age were analyzed on a single immunoblot using antibodies against the calpain recognition sequence in spectrin. Rat brain membranes expressing calpain-induced spectrin fragmentation were included in lanes 1 and 2 as positive controls (6 and 80 µg protein, respectively). The 150-kDa BDP_N antigen was sufficiently immunolabeled for image analysis while no labeling occurred to the parent spectrin (S) whose electrophoretic position was subsequently determined by anti-spectrin staining. Lower panel: BDP_N in samples from nine young mice and eight middle-aged mice was quantitatively compared within single blots using integrated densitometry. Each bar represents the mean immunoreactivity ± SEM. Two-tailed Mann-Whitney U-test: *p=0.01, **p=0.005. O, olfactory bulb; N, neocortex; H, hippocampus; T, thalamic-rich tissue; C, cerebellum and brain stem.
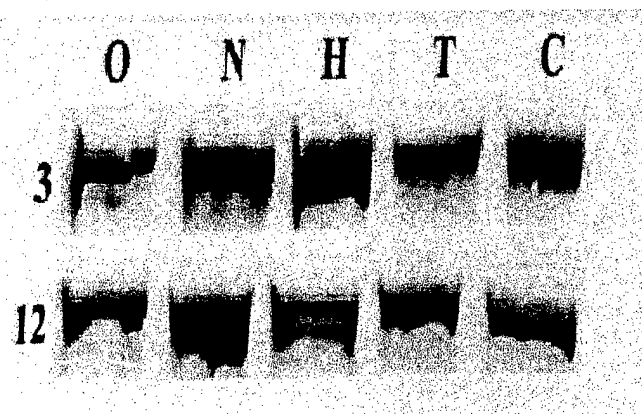
Figure 2. Focal localization of calpain-mediated spectrin breakdown is evident in the cortex of middle-aged mice. Brain sections from 3- (a) and 12-month-old mice (b) were subjected to immunocytochemical staining with antibodies that specifically recognize the calpain-mediated BDP_N antigen. The photomicrographs show that patterned, punctate immunoreactivity is present in the 12-month tissue along cortical neurons (see arrows). Scale bar: 30 μm.
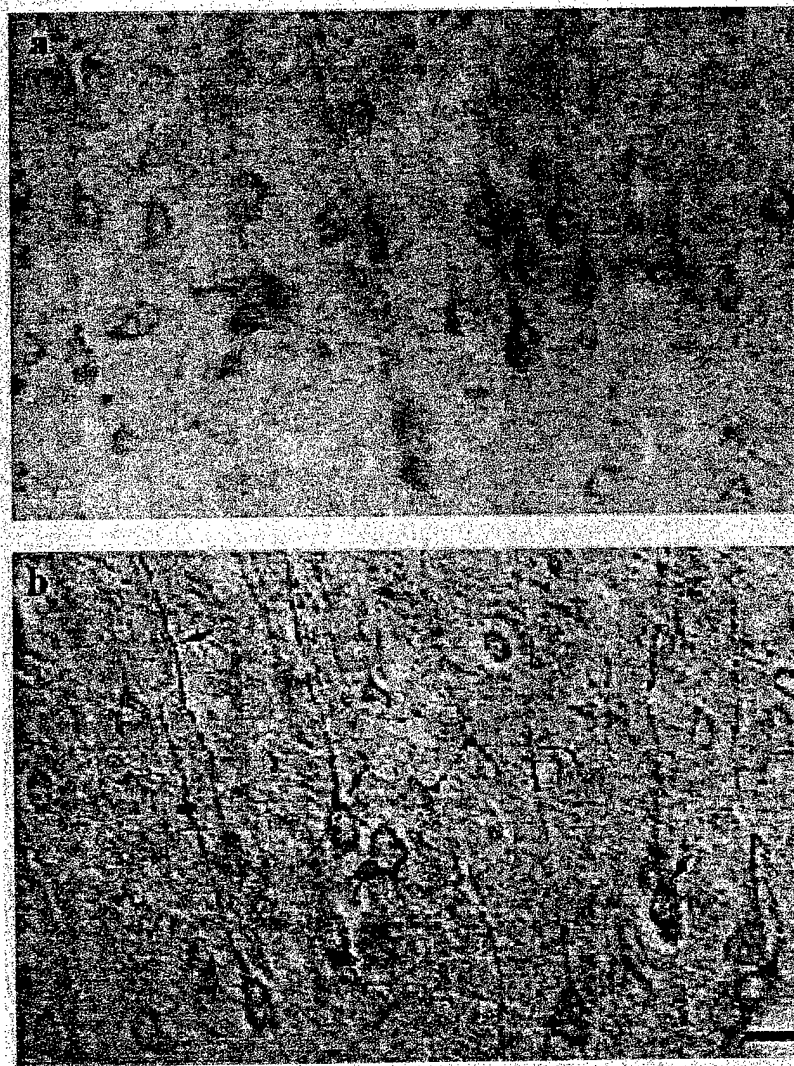
Figure 3. Calpain-mediated spectrin breakdown product selectively accumulates in field CA1 of the hippocampus from middle-aged mice. Brain sections from 3- (a, c) and 12-month-old mice (b, d) were immunostained with antibodies that specifically recognize the calpain-mediated spectrin fragment BDP_N. Photomicrographs show the pyramidal zone of the CA1 subfield (a, b) and the regions of CA3 and dentate gyrus (c, d). sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: a and b, 50 μm; c and d, 100 μm.

Figure 4 (below). Calpain I-mediated spectrin breakdown product selectively accumulates in field CA1 of the hippocampus from middle-aged mice. Brain sections from 3- (a, c) and 12-month-old mice (b, d) were immunostained with antibodies that specifically recognize the calpain-mediated spectrin fragment BDP_N. Photomicrographs show the pyramidal zone of the CA1 subfield (a, b) and the regions of CA3 and dentate gyrus (c, d). sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: a and b, 50 μm; c and d, 100 μm.

POSITIVE MODULATION OF AMPA-TYPE GLUTAMATE RECEPTORS ELICITS NEUROPROTECTION AFTER TRIMETHYLTIN EXPOSURE IN HIPPOCAMPUS

Subramani Munirathinam[1,2], Gary Rogers[3], and Ben A. Bahr[1,2]

[1]Department of Pharmaceutical Sciences and the Neurosciences Program, [2]Center for

Drug Discovery, University of Connecticut, Storrs, Connecticut, USA;

[3]Cortex Pharmaceuticals, Irvine, California, USA

Running title: Neuroprotection through AMPA receptor modulation

Correspondence to:

Ben A. Bahr

Dept. of Pharmaceutical Sciences and the Neurosciences Program

University of Connecticut, Storrs, CT 06269-2092

Tel: 860-486-6043; Fax: 860-486-6858

E-mail: Bahr@uconn.edu

ABSTRACT

Positive Modulation of AMPA-Type Glutamate Receptors Elicits Neuroprotection after Trimethyltin Exposure in Hippocampus. Munirathinam, S., Rogers, G., and Bahr, B. A. (2002). *Toxicol. Appl. Pharmacol.* **000**, 000-000.

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamatergic receptors have been linked to survival signaling, especially when the receptors are allosterically modulated by members of the Ampakine family. While increased glutamatergic communication through AMPA receptors has been shown to protect against toxic conditions that target hippocampal subfield CA1, protection in other subfields has not been shown. Accordingly, positive modulation of AMPA receptors by Ampakine compounds CX727 and CX516 was tested for effects on trimethyltin (TMT) neurotoxicity in rat hippocampal slice cultures. TMT was applied for 4 h followed by a rapid washout and antagonistic quenching of AMPA and N-methyl-D-aspartate (NMDA) receptors. After a 24-h period, the TMT-exposed slices exhibited increased levels of calpain-mediated spectrin breakdown as well as synaptic deterioration. TMT selectively targeted CA3 pyramidal neurons and dentate gyrus (DG) granule cells as evidenced by degeneration and neuronal loss. The cytoskeletal and synaptic damage was reduced when Ampakine modulation was initiated during the post-insult period. Furthermore, the extent of protection was comparable to that produced by the NMDA receptor antagonist AP5. The above results were substantiated by histological experiments, revealing that Ampakine treatment prevented TMT-induced cell loss in CA3 and DG. These results indicate that AMPA receptor signals are part of cellular repair responses following exposure to an environmental toxin.

Key words: Ampakines, BDP, calpain, CX516, excitotoxicity, hippocampal slice culture, spectrin breakdown product

INTRODUCTION

AMPA-type glutamate receptors are known to participate in higher cognitive functions in the mammalian brain. Positive modulation of AMPA receptors by the Ampakine class of compounds improves channel function by making the receptors more responsive to endogenous ligand and specific agonists (Tang et al., 1991; Arai et al., 1996; Suppiramaniam et al., 2001). Positive modulators in the Ampakine family of benzamide derivatives act selectively on AMPA receptors. The resultant increase in glutamatergic transmission has been shown to be associated with enhanced synaptic plasticity and memory retention (Staubli et al., 1992, 1994; Granger et al., 1996; Hampson et al., 1998; Lebrun et al., 2000).

Excessive glutamatergic activity through AMPA receptors is known to facilitate excitotoxic damage, and negative modulation of the receptor activity through antagonists is well known to be protective (Buchan et al., 1991; Nellgard and Wieloch, 1992; Sheardown et al., 1993). Interestingly, low-level stimulation of AMPA receptors by endogenous glutamate has been shown to enhance neuronal survival and promote synaptic maintenance (Bambrick et al., 1995; McKinney et al., 1999). This is likely related to the fact that besides having ionotropic properties, AMPA receptors are linked to the neuroprotective mitogen-activated protein kinase (MAPK) pathway (Wang and Durkin, 1995; Hayashi et al., 1999; Bahr et al., 2002; Limatola et al., 2002). This signaling pathway can be positively modulated (Bahr et al., 2002), thereby implicating it in Ampakine induction of neurotrophic factor expression (Lauterborn et al., 2000) and neuroprotection against stroke-type excitotoxicity (Bahr et al., 2002). Thus, AMPA receptors are part of a cellular mechanism(s) that is necessary for both information processing and compensatory signals in response to injury.

In the present study, we tested whether enhanced glutamatergic signaling elicits protection across different hippocampal subfields. Thus, AMPA receptor modulation was tested

for neuroprotection against trimethyltin (TMT) exposure. TMT is an organotin molecule that has been used to model selective brain damage (Dyer et al., 1982; Chang, 1996; Ishida et al., 1997; Noraberg et al., 1998; Philbert et al., 2000). The potent neurotoxin targets vulnerable neurons and is known to cause amnesia and selective damage in the hippocampus, possibly through excitotoxic mechanisms (Brodie et al., 1990; Feldman et al., 1993; Bahr et al., 1995b; Dawson et al., 1995). TMT exposure causes seizures in animal models and humans (Feldman et al., 1993; Ishida et al., 1997), as well as severe deficits in learning and memory (Kreyberg et al., 1992; Ishida et al., 1997; Ishikawa et al., 1997). At the molecular level, TMT exposure leads to distinct changes in growth factor and activity-dependent gene expression (Andersson et al., 1997), evidence of cytoskeletal damage (Bahr et al, 1995b, 2002), and selective loss of adhesion molecules (Dey et al., 1994) and synaptic markers (Harry et al., 1985; Brock and O'Callaghan, 1987; Bahr et al., 2002). The hippocampal slice model was used since the hippocampus is a brain area that has been extensively utilized to study TMT toxicity. It is advantageous to use the slice model because of its features characteristic of the adult brain including the circuitry, integrity, and organization of neuronal subfields (Bahr et al., 1995a). Neurotoxicity measures of particular interest were the loss of synaptic proteins and the activation of the calcium-dependent protease calpain. The decline in expression of synaptic markers represents early neurodegeneration and is useful for identifying potential compensatory pathways (Harry et al., 1985; Brock and O'Callaghan, 1987; Bahr et al., 1994, 1998, 2002; Bendiske et al., 2002; Bendiske and Bahr, 2002). Many types of toxic insults are known to promote calpain-mediated degradation of the cytoskeleton, a sensitive and very early marker of pathology (see Vanderklish and Bahr, 2000). Experiments were conducted to study the neuroprotective effect of two positive modulators of AMPA receptors, Ampakine CX516 [1-(quinoxalin-6-ylcarbonyl)piperidine] and the related compound CX727. CX727 is a metabolically stable analog of the Ampakine CX516.

4

METHODS

Hippocampal slice preparation. Sprague-Dawley rat litters (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health. Animals 11-12 days postnatal were quickly treated with isoflurane anesthesia then decapitated to prepare organotypic hippocampal slices. The brains were rapidly removed and cooled in ice-cold buffer containing 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 4 mM $MgSO_4$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM ascorbic acid and 75 µM adenosine (pH 7.2). Hippocampi were collected and 400-µm transverse slices were prepared starting from the septal to the temporal end. Groups of 7-8 slices were distributed onto Biopore insert membranes (Millicell-CM, Millipore Co., Bedford, MA), which remained in contact with the culture media. The media included 50% Basal Medium Eagle, 25% Earl's salt solution, 25% horse serum, and supplemented to the following final concentrations: 136 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 4 mM $NaHCO_3$, 3 mM glutamine, 40 mM glucose, 0.4 mM ascorbic acid, 20 mM HEPES buffer (pH 7.3 at 23 °C), 1 mg/L insulin (24 I.U. per mg), 5 units/ml penicillin, and 5 mg/l streptomycin. The slices were maintained for 15-20 days at 37 °C in a humid incubator supplied with 5% CO_2, with the media being changed every 2-3 days.

Treatment schedule. Freshly prepared medium was changed one day before the actual experiment. Slices were treated with 100 µM TMT (Aldrich Chemical Co., Milwaukee, WI) in serum-free media for 1-4 h. The slices were then washed twice (5 min each) with media containing 20 µM MK801 and 40 µM CNQX (Tocris, Ballwin, MO) in order to establish a controlled, acute toxic exposure. Glutamate receptor antagonists inhibit the progressive cellular changes induced by TMT as previously shown (Bahr et al., 1995b). Groups of TMT-treated slices were allowed to recover in media containing serum for 24 h in the presence of Ampakine

5

compounds (0-100 μM). Ampakine compounds were used at the respective concentrations that produced mild to maximal increases in electrophysiological response size measured in hippocampal slices (e.g., see Arai et al., 1996). A separate group of slices were co-exposed with TMT and the NMDA receptor antagonist AP5 (TOCRIS, Ballwin, MO) for 4 h. All treatment groups were harvested after the 24-h recovery period.

Immunoblot analyses. Immunoblot technique was carried out as described (Bahr et al., 1995b, 2002). Slice cultures prepared from 25 brains were harvested in ice-cold homogenization buffer containing 0.32 M sucrose, 5 mM HEPES buffer (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 10 μg/l antipain, and 2 μg/l each of leupeptin, aprotinin, and pepstatin. In groups of 6-8, the slices were gently harvested using a soft brush. Slice samples were then subjected to one freeze-thaw cycle and homogenized by sonication in 50 μl of hyposomotic lysis buffer containing 8 mM HEPES buffer (pH 7.4), 1 mM EDTA, 0.3 mM EGTA, and protease inhibitors listed above. Protein concentration was determined with BSA as the standard. Equal aliquots of the slice samples (85 μg protein) were prepared in 2.5% (wt/vol) SDS in the presence of 3% (vol/vol) β-mercaptoethanol and incubated at 100 °C for 5 min. The denatured samples were then subjected to electrophoresis on polyacrylamide gradient gels (4-16% wt/vol) and transferred to nitrocellulose (0.2 μm pore size) for 12 h. Immunodetection of transferred proteins used the following antibodies diluted in 1.5% nonfat dry milk: anti-GluR1 prepared as described (Bahr et al., 1996), anti-synaptophysin (Boehringer Mannheim, Indianapolis, IN), and anti-spectrin BDP_N (Bahr et al., 1995b). The nitrocellulose membrane was incubated with primary antibodies at 4°C overnight with gentle agitation. Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates. For color development, the 5-bromo-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system was used. Color development was stopped prior to

6

maximal intensity to avoid saturation, and single blots were used for comparative studies between different antigens. Integrated densities of the labeled antigens were quantitatively compared within single immunoblots using software from Bioquant Image Analysis System (R&M Biometrics,Inc., Nashville, TN).

Tissue staining by Nissl. Cultured hippocampal slices prepared from 10 brains were fixed with 4% paraformaldehyde for 2 h, then fixative was removed and replaced with 20% sucrose for 1 h. Sections of 20-μm thickness were cut and stained with cresyl violet.

Statistics. Data were expressed as mean ± SEM and statistical significance was determined by one-way analyses of variance followed by the Tukey-Kramer multiple range tests. Differences were considered significant at $p < 0.05$.

RESULTS

TMT Toxicity in Hippocampal Slice Cultures

The toxic action of TMT was assessed in long-term hippocampal slice cultures. Time-dependent changes were found in markers of cytoskeletal and synaptic components after exposure to 100 μM TMT. TMT was used at a sufficient level to elicit acute toxicity in a rapid manner throughout the multiple cell layers of the organotypic cultures (see Bahr et al., 1995a). Cleavage of the cytoskeletal protein spectrin via the protease calpain increased over time of TMT exposure (Fig. 1A). The amino-terminal spectrin breakdown product (BDP_N) of 145 kDa was labeled in immunoblot samples with antibodies directed against the calpain recognition site. The TMT insult of 1 h caused calpain activation, and the BDP response was still evident 5 h post-

7

insult (solid line). This BDP signal dissipated by 24 h post-insult (dotted line), concluding that the tissue can exhibit recovery from short toxin exposure. However, longer TMT treatment of 4 h caused sustained pathogenic activation of the protease that continued to increase over the 24 h recovery period. Similar evidence of sustained neurodegeneration after a 4-h TMT exposure was found by measuring the postsynaptic marker GluR1 (Fig. 1B) and the presynaptic marker synaptophysin (see Fig. 2B).

Positive Modulation of AMPA Receptors Promotes Cytoskeletal and Synaptic Repair

Next, we tested positive modulators of AMPA receptors for neuroprotection in hippocampal slice cultures previously exposed to TMT for 4 h. Allosteric modulators of the glutamate receptors, termed Ampakines, have been previously shown to promote cell survival through the link between AMPA receptors and MAPK signaling (Bahr et al., 2002). The Ampakine CX727 was infused into the slices for the 24-h post-insult period, after which slice samples were prepared and analyzed for the calpain BDP response (Fig. 2A), synaptophysin (Fig. 2B), and GluR1 (Fig. 3A). The molecular markers indicated protection when comparing immunoblot samples from TMT-exposed slices (n=18-27 groups of 7-8 slices each) with those that received CX727 after the insult (n=12 groups). The Ampakine CX727 reduced the TMT-mediated spectrin breakdown in a dose-dependent manner, completely eliminating the calpain activation event at the higher dose (post-hoc Tukey test: $p<0.001$). Correspondingly, the neuroprotectant action restored the pre- and postsynaptic proteins to levels comparable to those found in control slices (n=17-24 untreated groups). Similar to the cytoskeletal protection, Figure 2B shows that the higher dose of CX727 also caused complete restoration of the presynaptic marker ($p<0.001$). At the lower CX727 dose of 20 µM, TMT-induced spectrin proteolysis was

reduced by 37%, synaptophysin exhibited 39% recovery, and GluR1 exhibited 51% recovery (post-hoc tests: $p<0.001$, $p<0.05$, and $p<0.001$, respectively).

The observed neuroprotection associated with post-insult Ampakine treatment was compared to that produced by the known blocker of excitotoxicity AP5. AP5 is an NMDA receptor antagonist that has previously been shown to block pathogenic calpain activation induced by TMT (Bahr et al., 1995b). When 100 µM AP5 was present during both the 4-h insult and the recovery period, the TMT-induced spectrin breakdown measure of 36 ± 2.2 was significantly reduced to 4.4 ± 2.1 ($p<0.0001$). This is the same level of BDP reduction as that produced by the post-insult application of the Ampakine CX727 (Table 1). AP5 also caused pronounced restoration of the synaptic marker GluR1 (Fig. 3B). Similar levels of synaptic protection were elicited by CX727 (see Fig. 3A; post-hoc test: $p<0.001$) when applied during the recovery period.

Positive Modulation of AMPA Receptors Promotes Cell Survival

CX516 appears to be more effective than CX727 with regards to protecting against TMT-induced calpain activation and resultant spectrin breakdown. Evidence of spectrin proteolysis was completely abolished by Ampakine CX516 at 20 µM in TMT-treated slices, whereas 100 µM CX727 was required for such a level of neuroprotection (Table 1). Note that Arai et al. (1996) have demonstrated that 20 µM CX516 is comparable to the concentration needed to increase glutamatergic communication in hippocampal circuitries. Blocking cytoskeletal damage after excitotoxic protease activation is an important factor for cellular recovery since a correspondence exists between the extent of calpain-mediated spectrin breakdown and cell death (Bahr et al., 2002). Due to its potent action against spectrin breakdown, CX516 was tested for its ability to prevent neuronal loss in vulnerable regions. In addition to cytoskeletal damage and

9

synaptic decline, hippocampal slice cultures exposed to TMT for 4 h exhibited regionally selective cell death. Cresyl violet-stained sections revealed a marked decrease in CA3 pyramidal neurons and dentate gyrus granule cells (Fig. 4, middle panels) as compared to sections from control slices (top panels). Pyknotic alterations are evident in the remains of the CA3 subfield, and the dentate gyrus consists almost entirely of pyknotic nuclei in TMT-treated slices. The Ampakine modulator CX516 was applied post-insult, resulting in dramatic prevention of the pyknotic changes and cell death (Fig. 4, bottom panels).

DISCUSSION

The data presented show that for a prolonged period following TMT treatment, the exposed hippocampal tissue exhibited neuronal damage including cytoskeletal and synaptic deterioration. The latter correlates with previous *in vivo* studies in which the toxic action of TMT was shown to cause a marked loss of synaptic components (Harry et al., 1985; Brock and O'Callaghan, 1987; Whittington et al., 1989). TMT-treated hippocampal slice cultures provide a convenient model of selective neurodegeneration. The regional selectivity of the TMT-induced damage in the slice model was similar to that found in adult rats (Andersson et al., 1997). Interestingly, TMT action, *in vitro* and *in vivo*, targets the same subfield found to be particularly vulnerable to kainate-induced seizure activity (Nadler et al., 1980; Bi et al., 1996).

As with seizure responses, the TMT toxicity in the slice model involves glutamate since an NMDA receptor antagonist elicited cytoskeletal and synaptic protection. Glutamate excitotoxicity has been suggested to play a role in the toxic action of TMT (Patel et al., 1990; Bahr et al., 1995b; Dawson et al., 1995; Patterson et al., 1996; Ishida et al., 1997). However, TMT appears to express dual mechanisms of neurotoxicity with differential involvement of excitotoxic events. Low concentrations of TMT caused apoptotic death of cerebellar granule cells through oxidative

10

signaling, and higher TMT levels initiated necrotic death mediated by glutamate receptors (Gunasekar et al., 2001). Excitotoxicity was found to contribute little to the TMT-induced apoptosis in the granule cells. The concentration of TMT used in the present report suggests that the necrotic mode of cell death occurs in the hippocampal slice model. This is consistent with the pyknotic changes found in the dentate gyrus granule cell layer and in the CA3 pyramidal zone. NMDA receptors are a primary focus of studies on excitotoxicity (Lynch and Guttmann, 2002), and blocking these receptors in the slice model caused a marked reduction in neurodegeneration. In contrast, blockage of NMDA receptors did not reduce TMT-mediated apoptosis in cerebellar granule cells (Gunasekar et al., 2001).

Results from the slice model indicate that cellular repair is elicited by the positive modulation of glutamatergic activity through AMPA receptors. Enhanced glutamatergic activity has previously been shown to increase synaptic resilience and neuronal survival *in vitro* and *in vivo* (Bambrick et al., 1995; McKinney et al., 1999; Bahr et al., 2002; Limatola et al., 2002). Our current results clearly show that, through allosteric modulation, AMPA receptor responses can be intensified in such a way as to strengthen survival signaling and reduce hippocampal damage. The allosteric modulators used are known to selectively act on the AMPA class of glutamate receptors, leading to distinct changes in channel properties (see Staubli et al., 1992; Suppiramaniam et al., 2001). Previous studies identified a functional link between AMPA receptors and MAPK, and the work indicated that this pathway represents a compensatory response to CA1-targeting excitotoxins (Bahr et al., 2002). The present work found that the survival system activated with Ampakine modulators also protects against CA3-targeting neurotoxin exposure. Thus, the glutamate-mediated survival response can be exploited to attenuate the pathogenic sequela set in motion by the action of neurotoxins. It stands to reason

11

that, following toxin exposure, glutamatergic communication between affected neurons appears is vital for cellular maintenance signals.

Survival signaling through AMPA receptors may involve brain-derived neurotrophic factor (BDNF). AMPA receptor activation has been shown to increase BDNF mRNA (Hayashi et al., 1999) in a manner that can be positively influenced by Ampakine modulation on the same time scale as shown here for neuroprotection (Lauterborn et al., 2000). BDNF is a well-known component of injury-induced compensatory responses. In fact, the endogenous pathway that activates BDNF expression may explain why the CA1 region is not affected by TMT exposure (Andersson et al., 1997; also see Xiao et al., 1999). The CA3 region does not exhibit a compensatory change in BDNF production and, accordingly, is vulnerable to degeneration in the TMT-treated rat. It is conceivable that the AMPA receptor signaling pathway promotes those endogenous self-repair mechanisms that alone are not sufficient to protect vulnerable neurons against a toxic insult.

REFERENCES

Andersson, H., Wetmore, C., Lindqvist, E., Luthman, J., and Olson, L. (1997). Trimethyltin exposure in the rat induces delayed changes in brain-derived neurotrophic factor, fos and heat shock protein 70. Neurotoxicol. 18, 147-159.

Arai, A., Kessler, M., Rogers, G., and Lynch, G. (1996). Effects of a memory enhancing drug on DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents and synaptic transmission in hippocampus. J. Pharmacol. Exp. Ther. 278, 627-638.

Bahr, B. A., Abai, B., Gall, C., Vanderklish, P. W., Hoffman, K. B., and Lynch, G. (1994). Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp. Neurol. 129, 81-94.

Bahr, B. A., Kessler, M., Rivera, S., Vanderklish, P. W., Hall, R. A., Mutneja, M. S., Gall, C., and Hoffman, K. B. (1995a). Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5, 425-439.

Bahr, B. A., Tiriveedhi, S., Park, G. Y., and Lynch, G. (1995b). Induction of calpain-mediated spectrin fragments by pathogenic treatment in long-term hippocampal slices. J. Pharmacol. Exp. Ther. 273, 902-908.

Bahr, B. A., Hoffman, K. B., Kessler, M., Hennegriff, M., Park, G. Y., Yamamoto, R. S., Kawasaki, B. T., Vanderklish, P. W., Hall, R. A., and Lynch, G. (1996). Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. Neuroscience 74, 707-721.

Bahr, B. A., Hoffman, K. B., Yang, A. J., Hess, U. S., Glabe, C. G., and Lynch, G. (1998). Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J. Comp. Neurol. 397, 139-147.

Bahr, B. A., Bendiske, J., Brown, Q. B., Munirathinam, S., Caba, E., Rudin, M., Urwyler, S., Sauter, A., and Rogers, G. (2002). Survival signaling and selective neuroprotection through glutamatergic transmission. Exp. Neurol. 174, 37-47.

Bambrick, L. L., Yarowsky, P. J., and Krueger, B. K. (1995). Glutamate as a hippocampal neuron survival factor: an inherited defect in the trisomy 16 mouse. Proc. Natl. Acad. Sci. U.S.A. 92, 9692-9696.

Bendiske, J., and Bahr, B. A. (2002). Activation of the lysosomal system: A compensatory response against protein deposition and associated synaptopathogenesis. J. Neuropathol. Exp. Neurol., in press.

Bendiske, J., Caba, E., Brown, Q. B., and Bahr, B. A. (2002). Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. J. Neuropathol. Exp. Neurol. 61, 640-650.

Bi, X., Chang, V., Siman, R., Tocco, G., and Baudry, M. (1996). Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. Brain Res. 726, 98-108.

Brock, T. O., and O'Callaghan, J. P. (1987). Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary acidic protein are associated with chemical-induced injury to the rat central nervous system. J. Neurosci. 7, 931-942.

Brodie, M. E., Opacka-Juffry, J., Peterson, D. W., and Brown, A. W. (1990). Neurochemical changes in hippocampal and caudate dialysates associated with early trimethyltin neurotoxicity in rats. Neurotoxicol. 11, 35-46.

Buchan, A. M., Li, H., Cho, S., and Pulsinelli, W.A. (1991). Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. Neurosci. Lett. 132, 255-258.

Chang, L. W. (1996). Toxico-neurology and neuropathology induced by metals. In Toxicology of

Metals (Chang, L.W., Ed.), pp. 511–535, CRC Press, New York, NY.

Dawson, R. Jr., Patterson, T. A., and Eppler, B. (1995). Endogenous excitatory amino acid release

from brain slices and astrocyte cultures by trimethyltin and other neurotoxic agents. Neurochem.

Res. 20, 847-858.

Dey, P. M., Graff, R. D., Lagunowich, L. A., and Rehul, K. R. (1994). Selective loss of the 180-kDa

form of neural cell adhesion molecule in hippocampus and cerebellum of the adult mouse

following trimethyltin administration. Toxicol. Appl. Pharmacol. 126, 69-74.

Dyer, R. S., Deshields, T. L., and Wonderlin, W. F. (1982).Trimethyltin-induced changes in gross

morphology of the hippocampus. Neurobehav. Toxicol. Teratol. 4, 141-147.

Feldman, R. G., White, R. R., and Eriator, I. I. (1993). Trimethyltin encephalopathy, Arch. Neurol.

50, 1320-1324.

Granger, R., Deadwlyer, S., Davis, M., Moskovitz, B., Kessler, M., Rogers, G., and Lynch, G.

(1996). Facilitation of glutamate receptors reverses an age-associated memory impairment in

rats. Synapse 22, 332-337.

Gunasekar, P., Li, L., Prabhakaran, K., Eybl, V., Borowitz, J. L., and Isom, G. E. (2001).

Mechanisms of the apoptotic and necrotic actions of trimethyltin in cerebellar granule cells.

Toxicol. Sci. 64, 83-89.

Hampson, R. E., Rogers, G., Lynch, G., and Deadwyler, S. A. (1998). Facilitative effects of the

ampakine CX516 on short-term memory in rats: correlations with hippocampal neuronal activity.

J. Neurosci. 18, 2748-2763.

Harry, G. J., Goodrum, J. F., Krigman, M. R., and Morell, P. (1985). The use of synapsin I as a

biochemical marker for neuronal damage by trimethyltin. Brain Res.326, 9-18.

Hayashi, T., Umemori, H., Mishina, M., and Yamamoto, T. (1999). The AMPA receptor interacts

with and signals through the protein tyrosine kinase Lyn. Nature 397, 72-76.

Ishida, N., Akaike, M., Tsutsumi, S., Kanai, H., Masui, A., Sadamatsu, M., Kuroda Y., Watanabe, Y., McEwen, B. S., and Kato, N. (1997). Trimethyltin syndrome as a hippocampal degeneration model: temporal changes and neurochemical features of seizure susceptibility and learning impairment. Neuroscience 81, 1183-1191.

Ishikawa, K., Kubo, T., Shibanoki, S., Matsumoto, A., Hata, H., and Asai, S. (1997). Hippocampal degeneration inducing impairment of learning in rats: model of dementia?. Behav. Brain Res. 83, 39-44.

Kreyberg, S., Torvik, A., Bjorneboe, A., Wiik-Larsen, and Jacobsen, D. (1992). Trimethyltin poisoning: report of a case with postmortem examination. Clin. Neuropathol. 11, 256-259.

Lauterborn, J. C., Lynch, G., Vanderklish, P., Arai, A., and Gall, C. M. (2000). Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. J. Neurosci. 20, 8-21.

Lebrun, C., Pilliere, E., and Lestage, P. (2000). Effects of S 18986-1, a novel cognitive enhancer, on memory performances in an object recognition task in rats. Eur. J. Pharmacol. 401, 205-212.

Limatola, C., Ciotti, M. T., Mercanti, D., Santoni, A., and Eusebi, F. (2002). Signaling pathways activated by chemokine receptor CXCR2 and AMPA-type glutamate receptors and involvement in granule cells survival. J. Neuroimmunol. 123, 9-17.

Lynch, D. R., and Guttmann, R. P. (2002). Excitotoxicity: perspectives based on N-methyl-D-aspartate receptor subtypes. J. Pharmacol. Exp. Ther. 300, 717-723.

McKinney, R. A., Capogna, M., Durr, R., Gahwiler, B. H., and Thompson, S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat. Neurosci. 2, 44-49.

Nadler, J. V., Perry, B. W., Gentry, C., and Cotman, C. W. (1980). Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J. Comp. Neurol. 192, 333-359.

Nellgard, B., and Wieloch. T. (1992) Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. J. Cereb. Blood Flow Metab. 12, 2-11.

Noraberg, J., Gramsbergen, J. B. P., Fonnum, F., and Zimmer, J. (1998). Trimethyltin (TMT) neurotoxicity in organotypic rat hippocampal slice cultures. Brain Res. 783, 305 315.

Patel, M., Ardelt, B. K., Yim, G. K., and Isom, G. E. (1990). Interaction of trimethyltin with hippocampal glutamate. Neurotoxicol. 11, 601-608.

Patterson, T. A., Eppler, B., and Dawson, R. (1996). Attenuation of trimethyltin evoked glutamate (GLU) efflux from rat cortical and hippocampal slices. Neurotoxicol. Teratol. 18, 697-702.

Philbert, M. A., Billingsley, M. L., and Reuhl, K. R. (2000). Mechanisms of injury in the central nervous system. Toxicol. Pathol. 28, 43–52.

Sheardown, M. J., Suzdak, P.D., and Nordholm, L. (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. Eur. J. Pharmacol. 236, 347-353.

Staubli, U., Ambros-Ingerson, J., and Lynch, G. (1992). Receptor changes and LTP: An analysis using aniracetam, a drug that reversibly modifies glutamate (AMPA) receptors. Hippocampus 2, 49-58.

Staubli, U., Rogers, G. and Lynch, G. (1994). Facilitation of glutamate receptors enhances memory. Proc. Natl. Acad. Sci. U.S.A. 91, 777-781.

Suppiramaniam, V., Bahr, B. A., Sinnarajah, S., Owens, K., Rogers, G., Yilma, S., and Vodyanoy, V. (2001). Member of the Ampakines class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. Synapse 40, 154-158.

Tang, C-M., Shi, Q-Y., Katchman, A., and Lynch, G. (1991). Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. Science 254, 288-290.

Vanderklish, P. W., and Bahr, B. A. (2000). The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. Internatl. J. Exp. Pathol. 81, 323-339.

Wang, Y., and Durkin, J. P. (1995). α-Amino-3-hydroxy-5-methyl-4isoxazolepropionic acid, but not N-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein subunits in rat cortical neurons. J. Biol. Chem. 270, 22783-22787.

Whittington, D. L., Woodruff, M. L., and Baisden, R. H. (1989). The time-course of trimethyltin-induced fiber and terminal degeneration in hippocampus. Neurotoxicol. Teratol. 11, 21-33.

Xiao, Y., Harry, G. J., and Pennypacker, K. R. (1999). Expression of AP-1 transcription factors in rat hippocampus and cerebellum after trimethyltin neurotoxicity. Neurotoxicol. 20, 761-766.

Table 1.   Ampakine modulators and NMDA receptor antagonist produce similar levels of protection against TMT-induced cytoskeletal damage and synaptic decline.

Neuroprotectant	Reduction of Spectrin Breakdown	Restoration of GluR1
Ampakine CX516		
20 μM	99%	44%
100 μM	100%	70%
Ampakine CX727		
20 μM	37%	51%
100 μM	93%	79%
AP5		
100 μM	95%	74%

Levels of neuroprotection were determined from slice cultures previously exposed to TMT for 4 h. Neuroprotectants were infused into the slices for a 24-h post-insult period, after which the slices were harvested and assessed for changes in BDP and GluR1 by immunoblot.

FIGURE LEGENDS

Figure 1. Markers of cytoskeletal damage and synaptic decay illustrate the time course of TMT toxicity. Cultured hippocampal slices were treated with 100 μM TMT for 1 h or 4 h, then rapidly washed and quenched with CNQX and MK-801. The slices were maintained in culture during the 5-h (solid line) or 24-h (dotted line) post-insult period. Slices were harvested and samples were assessed in parallel by immunoblot. Integrated optical density levels of immunoreactivity (y-axis) were determined by image analysis (mean ± SEM), and the results obtained for spectrin BDP_N (A) and GluR1 (B) were plotted for 5-h (n = 6 groups of 7-8 slices each) vs. 24-h (n = 7) post-insult data. Immunoblot lanes represent slices treated with TMT for 1 h and harvested 24 h later (lane 1), and with TMT for 4 h and harvested 5 (lane 2) or 24 h later (lane 3).

Figure 2. Positive modulation of AMPA receptors promotes cytoskeletal and synaptic repair. Hippocampal slice cultures were untreated or exposed to 100 μM TMT for 4 h followed by rapid quenching of glutamate receptors. The 24 h post-insult recovery period was conducted in the presence of 0-100 μM CX727. Harvested slice samples were analyzed by immunoblot to determine integrated optical density levels of immunoreactivity for calpain-mediated spectrin BDP_N (A) and the presynaptic marker synaptophysin (B). Immunoblot lanes 1-4 correspond to the treatment order of the graphs. Each bar represents the mean integrated density ± SEM. Analyses of variance: A, $p < 0.0001$ and $F = 125$; B, $p < 0.0001$ and $F = 49.3$.

Figure 3. Ampakine CX727 and AP5 elicit similar synaptic protection against TMT exposure. Slices were untreated or exposed to 100 μM TMT for 4 h with the 24 h post-insult period including 20 or 100 μM CX727 (A). Alternatively, both the TMT exposure time and the post-insult period were conducted in the absence or presence of AP5 (B). Slice samples were analyzed by immunoblot to determine the integrated optical density levels for the AMPA receptor subunit GluR1. Immunoblot lanes 1-4 correspond to the treatment order in panel A. GluR1 immunoreactivity is expressed as mean integrated density ± SEM. Analyses of variance: A, $p < 0.0001$ and $F = 61.7$; B, $p < 0.0001$ and $F = 183$.

Figure 4. Positive modulation of AMPA receptors promotes cell survival. Cultured hippocampal slices were untreated (top panels) or exposed to 100 μM TMT for 4 h followed by a 24-h post-insult period in the absence (middle panels) or presence of 100 μM CX516 (bottom panels). Slices were then fixed, sectioned, and stained by Nissl. Low power photomicrographs show all neuronal subfields including the dentate gyrus (DG) and CA3 that are targeted by TMT (see arrows). High power photomicrographs are shown to illustrate neurodegeneration induced by TMT. Size bar: leftmost panels, 106 μm; remaining panels, 22 μm.
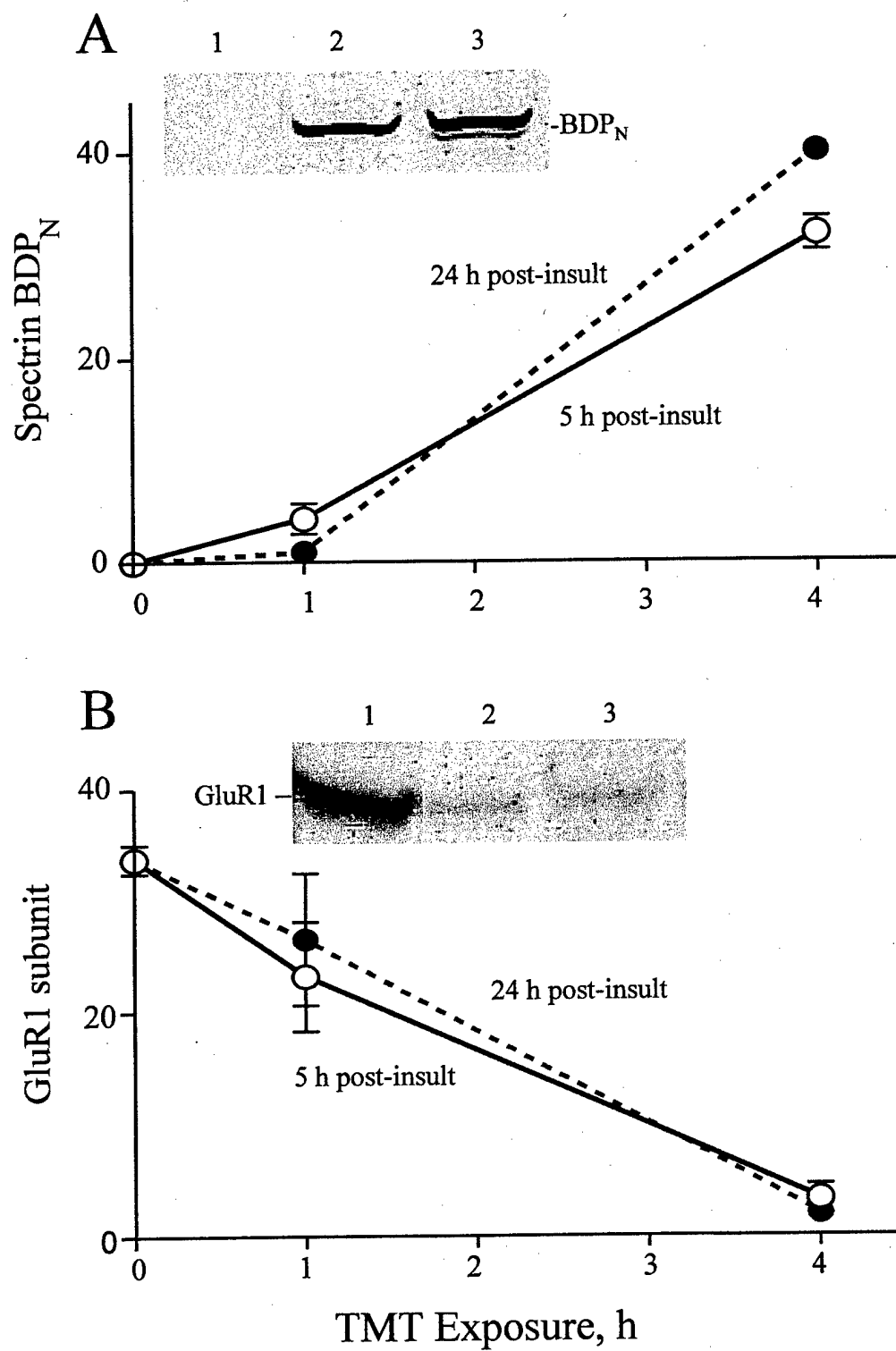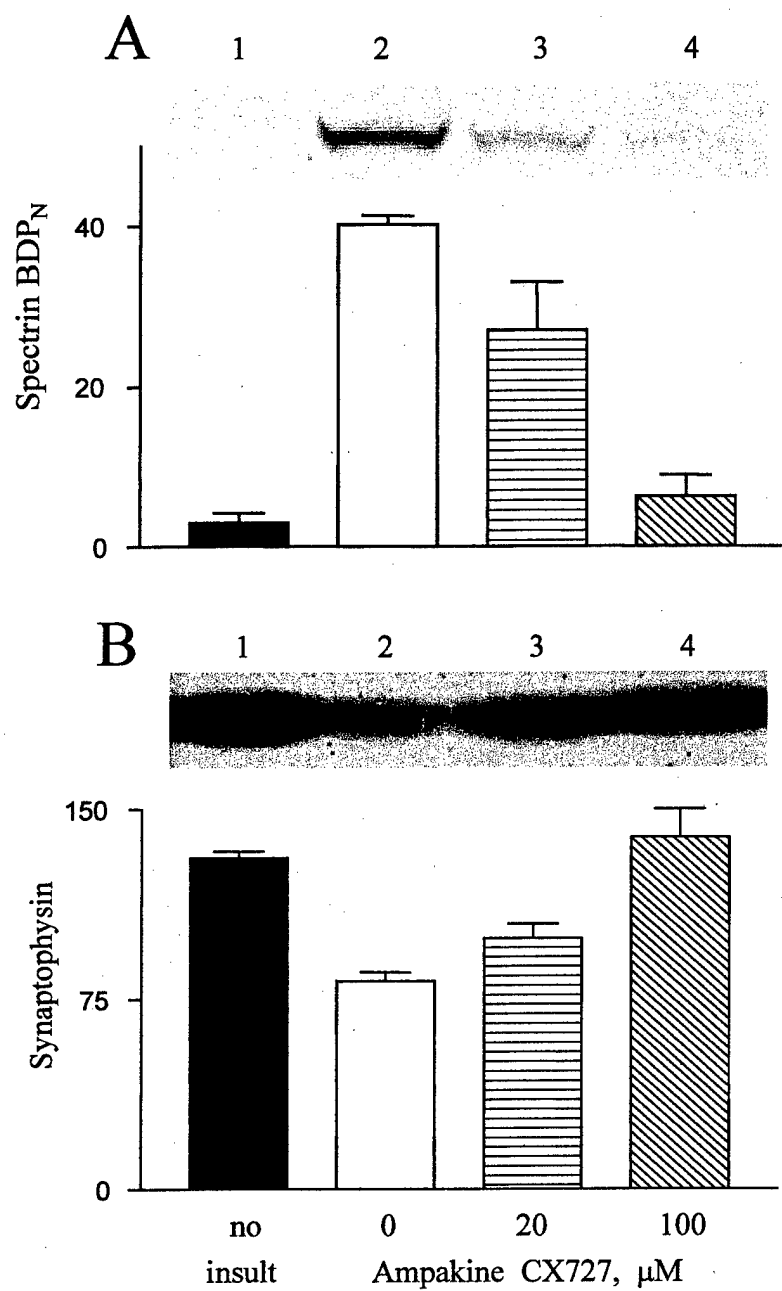
Figure 1

Figure 2

A

B

no insult

0   20   100

Ampakine CX727, μM

Figure 3

A

B

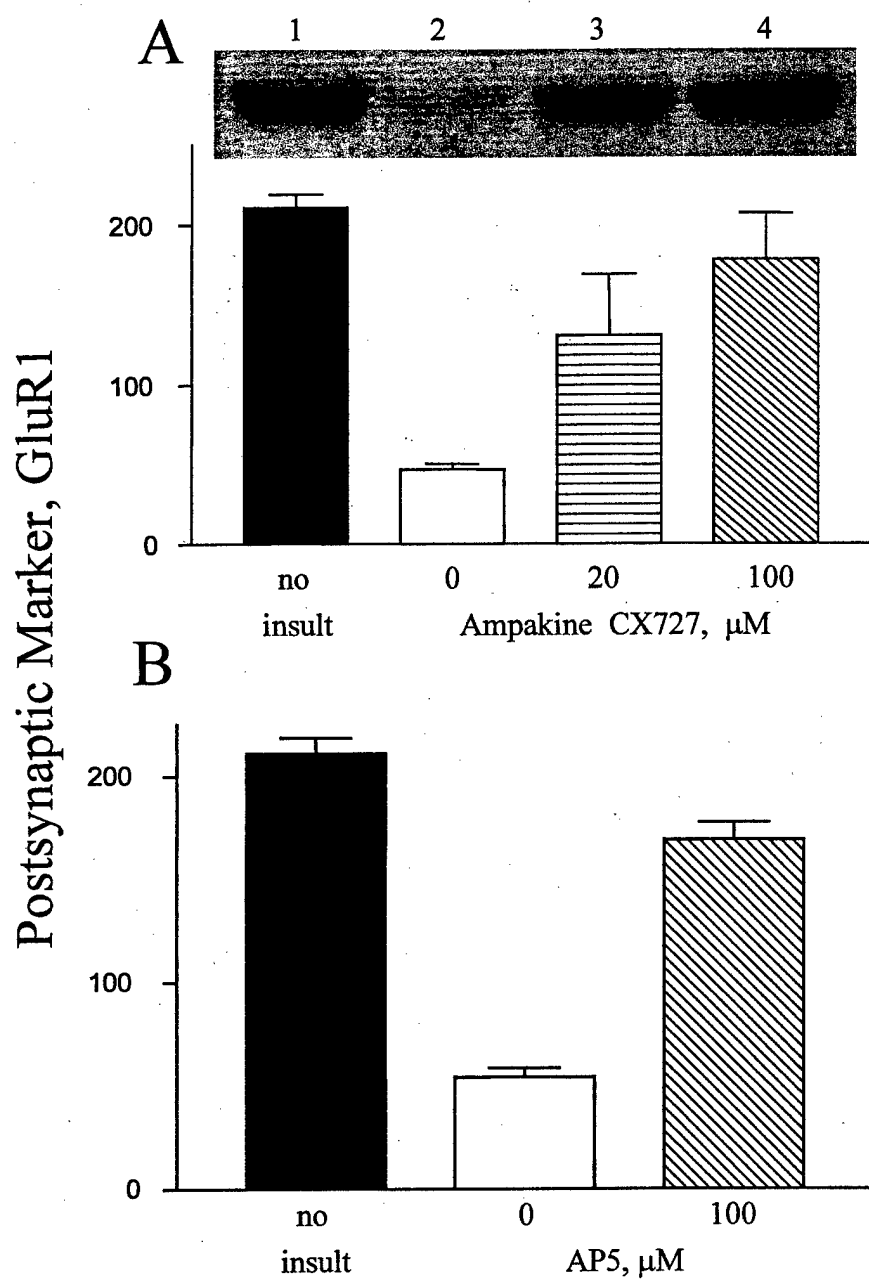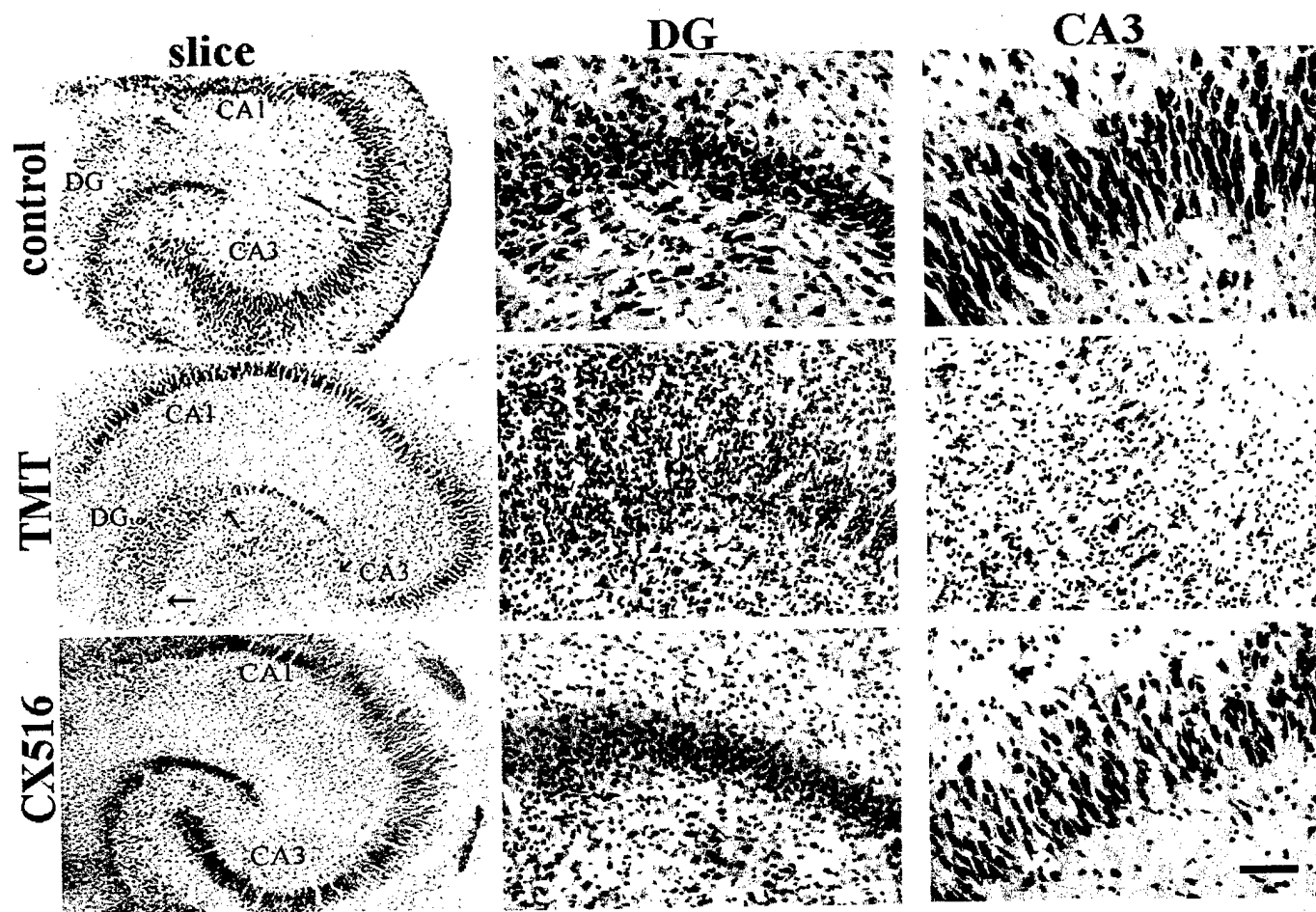
Postsynaptic Marker, GluR1

Figure 4

The Neuropathogenic Contributions of Lysosomal Dysfunction

Ben A. Bahr and Jennifer Bendiske

Department of Pharmaceutical Sciences and the Neurosciences Program

University of Connecticut

Storrs, Connecticut, U.S.A.

Abstract

Multiple lines of evidence implicate lysosomes in a variety of pathogenic events that produce neurodegeneration. Genetic mutations that cause specific enzyme deficiencies account for more than 40 lysosomal storage disorders. These mostly pre-adult diseases are associated with abnormal brain development and mental retardation. Such disorders are characterized by intracellular deposition and protein aggregation, events also found in age-related neurodegenerative diseases including i) Alzheimer's disease and related tauopathies, ii) Lewy body disorders and synucleinopathies such as Parkinson's disease, and iii) Huntington's disease and other polyglutamine expansion disorders. Of particular interest for this review is evidence that alterations to the lysosomal system contribute to protein deposits associated with different types of age-related neurodegeneration. Lysosomes are in fact highly susceptible to free radical oxidative stress in the aging brain, leading to the gradual loss of their processing capacity over the lifespan of an individual. Several studies point to this lysosomal disturbance as being involved in amyloidogenic processing, formation of paired helical filaments, and the aggregation of α-synuclein and mutant huntingtin proteins. Most notably, experimentally-induced lysosomal dysfunction, both *in vitro* and *in vivo*, recapitulates important pathological features of age-related diseases including the link between protein deposition and synaptic loss.

The endosomal-lysosomal pathway consists of a dynamic system of organelles working to recycle cellular ingredients, thereby providing a constant supply of basic components necessary to maintain the health of the cell. Many neurodegenerative diseases arise from lysosomal dysfunction, thus indicating the importance of these organelles for cell viability. Mutations that cause lysosomal enzyme deficiencies result in approximately 40 different syndromes. These diseases, termed lysosomal storage disorders (LSDs), have insidious effects with a time of onset ranging from *in utero* to 25 years of age (see Walkley, 1998). Reduced lysosomal capacity leads to the deposition of cellular material and the characteristic developmental problems.

Intracellular accumulation of aberrant protein and glycoconjugate species is believed to underlie the neurodegeneration as well as the clinical manifestations of LSDs including mental retardation, progressive cognitive decline, and behavioral inappropriateness. Similar phenotypic changes are common in diseases such as Alzheimer's disease (AD), frontotemporal dementia, and Parkinson's disease (PD). Interestingly, aberrant cellular accumulations also are shared by LSDs and age-related neurodegeneration, and evidence suggests that lysosomal disturbances play a role in both. Noted in Table 1, abnormal lysosome functioning is not only associated with the early-onset pathologies but also with the later-onset age-related diseases. In addition, lysosomes play a part in the early pathogenesis of prion encephalopathies (Laszlo et al., 1992). Cellular compromise is initiated at a critical level of lysosomal capacity, which is reduced with brain aging. Lysosomal instability appears to progress throughout the lifespan of an animal (see Brunk and Brun, 1972; Kikugawa et al., 1994; Nakamura et al., 1998). Gradual alterations to the lysosomal system with age may very well be the reason aging is the primary risk factor for many neurodegenerative diseases.

As in the case of LSDs, many of the later-onset disorders in Table 1 exhibit a degree of lysosome alteration, thus prompting studies addressing whether lysosomal disturbances contribute to age-related pathogenesis. This short review summarizes the evidence that lysosomal dysfunction plays a part in protein

aggregation events found in AD as well as in tauopathies linked to chromosome 17 mutations (FTDP-17). Lysosomes may also promote intracellular deposits that are hallmarks of other age-related disorders including PD and Huntington's disease (HD). The inherent toxicity of aggregated material and aggregated protein stress responses may be the fundamental component among protein deposition diseases (Bucciantini et al., 2002). Disturbance of lysosome functionality is not proposed as the cause of disease but rather implicated in the progression of neurodegeneration in conjunction with potentially several other events (e.g., see Small et al., 2001 for an excellent review of the pathogenic processes being investigated in the AD field).

Alzheimer-type protein deposition

Abnormal deposits of protein species are a hallmark of AD. Cellular processing pathways that degrade the amyloid precursor protein (APP) can lead to accumulation of toxic carboxyterminal fragments (CTFs) and Aβ peptides (e.g., see Oster-Granite et al., 1996; McPhie et al., 2001; Selkoe, 2001a). A wide body of work by Nixon and colleagues has implicated lysosomal abnormalities as a contributing factor in amyloidogenic accumulation and in the development of fibrillar Aβ in the form of senile plaques (Cataldo et al., 1991, 1994; Nixon et al., 2001). These studies and others point to changes in the lysosomal system of neurons as a prominent feature of AD pathology. Disturbances in the endosomal-lysosomal pathway were found to have a marked prevalence in AD-vulnerable regions like the prefrontal cortex and hippocampal pyramidal layers (Cataldo et al., 1996). The same study showed little evidence of such disturbances in the cerebellum, a less vulnerable region.

Age-related lysosomal perturbation has been previously suggested to promote pathogenic fibril formation and intracellular deposits of amyloidogenic species in AD brains (Benowitz et al., 1989; Cataldo et al., 1991). Alternative processing pathways through lysosomes were identified that potentially contribute to the production of amyloidogenic polypeptides (Cole et al., 1989; Golde et al., 1992; Haass et al., 1992, 1993; Koo and Squazzo, 1994; Evin et al., 1995; D'Andrea et

al., 2001). Correspondingly, alterations to the lysosomal system were particularly evident in a form of familial AD and in related transgenic mouse models (see recent review by Nixon et al., 2001). Administration of lysosomal inhibitors into brain ventricles of rats (Hajimohammadreza et al., 1994), systemically in mice (Mielke et al., 1997), and to brain tissue in culture (Bahr et al., 1994) confirmed the induction of Aβ-containing APP fragments in neurons. In addition, Aβ itself is internalized by AD-vulnerable neurons (Bahr et al., 1998) causing free radical generation within lysosomes and disruption of the lysosomal proton gradient (Yang et al., 1998; Ditaranto et al., 2001). The accumulation of stable Aβ in lysosomes promotes further production of amyloidogenic fragments (Yang et al., 1995; Bahr et al., 1998), perhaps as part of a pathogenic cascade (see Fig. 1).

There are several potential ways in which lysosomal disturbances might influence amyloidogenic events. From the cell surface, APP is reinternalized into the endosomal-lysosomal system where minor amounts may be converted to CTFs – these amyloidogenic intermediates would be long-lived when general hydrolase activity is suppressed by episodes of lysosomal perturbation. Compromised lysosomes are more likely to increase the half-life of CTFs generated by β-secretase processing in the endoplasmic reticulum (ER) and trans Golgi network (TGN), and the fragments would then buildup in neurons due to the reduced digestion/excretion activity of dysfunctional lysosomes. Another possibility is that abnormal endosomal-lysosomal pathways alter APP trafficking to promote availability for processing in the TGN. The resulting cellular accumulation of CTFs, as seen in experimental models (Golde et al., 1992; Bahr et al., 1994), would allow increased production of Aβ via γ-secretase cleavage. Lysosomal dysfunction could indirectly affect one or more of the myriad of processes that regulate APP catabolism (see Mills and Reiner, 1999). Since there is evidence of different γ-secretases for the generation of $A\beta_{1-42}$ and the less amyloidogenic $A\beta_{1-40}$ (Klafki et al., 1996; Skovronsky et al., 2000), one could speculate that altered compartmentation by lysosomal disturbance may encourage the action of $A\beta_{1-42}$-specific γ-secretase in order to

increase the $A\beta_{1-42}/A\beta_{1-40}$ ratio of non-secreted peptides and promote $A\beta$ fibrils. Mathews et al. (2002) recently proposed that abnormalities in the lysosome system lead to modified compartmentation of lysosomal hydrolases, conceivably resulting in accelerated $A\beta$ generation. Alternatively, preferential compartmentation that prevents γ-secretase activity may actually promote selective pathogenesis through CTFs (see McPhie et al., 2001; Amtul et al., 2002). Subsequent to CTF accumulation, the fragments may very well add to nucleated structures of intracellular $A\beta$, resulting in heterogeneous aggregation that facilitates the pathophysiology of dystrophic neurites (see Glabe, 2001). Finally, suppressed lysosome function could increase the cellular release of CTFs that would compete with and down-regulate $A\beta$ degradation/clearance pathways (see Iwata et al., 2001; Selkoe, 2001b), thereby promoting the deposition of the $A\beta$ peptide as amyloid plaques. It is not known as to which of these scenarios or combination thereof explains the amyloidogenic changes associated with lysosomal dysfunction. While there are many questions to be the focus of future research, mounting evidence indicates that the lysosomal disturbances found in the AD brain contribute to Alzheimer-type pathogenesis.

Suppression of lysosomal function not only promotes amyloidogenic events but also intraneuronal deposits recognized by antibodies against neurofibrillary tangles (NFTs). The microtubule-binding protein tau forms paired helical filaments (PHFs) leading to the production of NFTs in the aged brain and more so in patients with AD. Studies in rats and primates showed that inhibitors of lysosomal proteases induce the type of PHF aggregates that promote tangle formation (see Ivy, 1992; Takauchi and Miyoshi, 1995). *In vivo* and *in vitro* models have shown that altered processing of tau isoforms occur in response to lysosomal dysfunction, followed by intracellular deposition that resemble tangles at the light microscope (Bahr, 1995; Takauchi and Miyoshi, 1995; Bi et al., 2001; Bendiske et al., 2002) and electron microscope level (Takauchi and Miyoshi, 1995; Bi et al., 2001). Tau mutations cause NFTs in the absence of $A\beta$ plaques, resulting in a group of dementias called tauopathies or frontotemporal dementia (e.g., FTDP-17; see

Hardy et al., 1998). Thus, it is clear that abnormal tau processing alone is sufficient to cause neurodegeneration. Modified tau is thought to be metabolized in lysosomes (Ikeda et al., 1998, 2000). Along with the fact that $A\beta$ peptide is taken up by neurons and causes lysosomal perturbation (Bahr et al., 1998; Yang et al., 1998; Ditaranto et al., 2001) perhaps sheds light on the relationship between amyloid accumulation and tangle formation, a major issue in AD. A connection between $A\beta$ deposits and NFTs also was strengthened by recent studies using transgenic animals (Gotz et al., 2001; Lewis et al., 2001). One of the studies injected the longer, more amyloidogenic form $A\beta_{1-42}$ into tau mutant mice, causing increased tau tangles not in the hippocampal injection site but in a region to which hippocampal neurons project. This finding could be related to data that suggest $A\beta_{1-42}$ is taken up by vulnerable neurons and transported to distant synaptic terminals where local protein processing is subsequently disrupted (Bahr et al., 1998; also see Masliah and Licastro, 2000).

It needs to be noted that abnormal deposits do not have to be proteinaceous in order to have a negative impact on neurons. For instance, different types of glycosphingolipids accumulate in the brains of Tay-Sach's and Sandhoff's disease patients due to mutations in isoforms of the lysosomal enzyme β-hexosaminidase. The neurodegenerative disease Niemann-Pick type C is characterized by the buildup of lipoprotein-derived cholesterol in lysosomes due to mutations in the NPC1 protein (Loftus et al., 1997). Interestingly, the excessive lysosomal storage of cholesterol may in fact promote abnormal protein processing as evidenced by the appearance of PHF-tau in Niemann-Pick disease resembling that found in AD (Auer et al., 1995). This is consistent with reports suggesting that altered tau processing and early PHF formation involve lysosomes of AD brains and model systems (Ikeda et al., 1998, 2000; Oyama et al., 1998; Bendiske et al., 2002). Such negative effects on lysosomes would explain why cholesterol-lowering drugs reduce the incidence of AD-type dementia as well as reduce intra- and extracellular levels of $A\beta$ peptides (Fassbender et al., 2001). These results, together with those previously discussed, promote the idea that the gradual disruption of

3

lysosomes observed during brain aging contributes to protein deposition diseases.

Alzheimer-type synaptic pathology

The influence lysosomal dysfunction has on amyloidogenic and tangle chemistries may be the pathogenic step that leads to early pathology targeting synapses (see Fig. 1). As exemplified in Figure 2, lysosomal perturbation in hippocampal slice cultures causes dramatic reductions in synaptic proteins and in evoked excitatory responses (Bahr et al., 1994, 2002; Bendiske et al., 2002). The decline in synaptic markers and respective mRNA species correlated with the degree of induced protein deposition. Synaptopathogenesis may stem from Aβ processing since intracellular generation of amyloidogenic species has been shown to lead to the disruption of synapse structure and functionality (e.g., see Bahr et al., 1998; Hsia et al., 1999; Mucke et al., 2000; Kim et al., 2001a; Walsh et al., 2002). Similar declines in synaptic markers are characteristic of AD brains, thus implicating synaptic loss as a major and early contributor to the reduced cerebral activity and resultant cognitive impairment (Terry et al., 1991; Mesulam. 1999). A link between lysosomal dysfunction and synaptic pathology was indeed identified in AD brains (Callahan et al., 1999; Masliah and Licastro, 2000; also see Cross et al., 1986). In particular, indications of both lysosomal stress and synaptic decline were evident in tangle-bearing neurons but not in neighboring tangle-free neurons (Callahan et al., 1999). Disturbance of the lysosomal system appears to be especially detrimental to synapses since it increases synaptic vulnerability to other insults (Bahr et al., 1994) and its effects are not attenuated by endogenous repair systems (Bahr et al., 2002).

In both AD and the hippocampal slice model, diminished expression of synaptophysin and other synapse-related genes was found in neurons containing tau deposits (Callahan et al., 1999; Bendiske et al., 2002). Protein deposition has been shown to correspond with the severity of neurodegenerative diseases, thus explaining the identification of synaptic loss as a major correlate of cognitive decline. Results from the slice model indicate the link between aberrant protein accumulation and synaptic pathology involves the disruption of transport mechanisms that are vital for synapse maintenance (Bendiske

et al., 2002). Lysosomal dysfunction influences a cascade of pathogenesis consisting of anomalous production of CTFs, hyperphosphorylation of tau isoforms, and excessive ubiquitination of cellular deposits. Subsequent formation of PHFs may disrupt the equilibrium between tau and phospho-tau (pTau), thus lowering the availability of normal tau for stabilizing microtubules and their transport mechanisms (Fig. 1). Microtubule destabilization indeed followed lysosomal dysfunction, indicated by the selective loss of acetylated tubulin in the slice model and as occurs in PHF-containing neurons in AD (Hempen and Brion, 1996).

Over an extended period of lysosomal dysfunction, CTF and PHF accumulation corresponded with the degree of microtubule compromise (Bahr et al., 1994; Bendiske et al., 2002). Signs of microtubule destabilization in the hippocampal slice model included tubulin breakdown and diminished transport capability. Transport failure is likely mediated through the loss of microtubule integrity as well as through physical obstruction by protein deposits. Other studies suggest that amyloidogenic processing alone can stall kinesin-mediated transport mechanisms (see Kamal et al., 2001). Axonal transport appears to be particularly vulnerable to protein deposition as evidenced by the somatic accumulation of abnormal aggregates, filamentous inclusions, and lysosomes in the human brain (Hempen and Brion, 1996; Li et al., 2001).

α-Synuclein disorders

PD is another age-related disease that is associated with abnormal protein accumulation. PD and AD together represent the two most common forms of neurodegeneration, and there is increasing recognition of a relationship between the two disorders. Both exhibit intracellular deposition, thus, altered protein processing and ubiquitin disposal pathways have been implicated in the pathologies. Neuronal accumulations of eosinophilic inclusions termed Lewy bodies occur in subcortical neurons of PD brains, primarily composed of ubiquitin and α-synuclein (e.g., see Spillantini et al., 1997). The filamentous inclusions also are present in Lewy body dementia and in neocortical neurons in a variant of AD. An inherited defect that produces

mutations in α-synuclein leads to a familial form of PD. Expression of mutant α-synuclein in neurons led to lysosomal dysfunction and disruption of the ubiquitin-dependent proteolytic system (Stefanis et al., 2001). With respect to a relationship between AD and PD, it is of interest that aggregated α-synuclein has been localized to dystrophic neurites around amyloid plaques in AD (Masliah et al., 1996; El-Agnaf and Irvine, 2000). Thus, disruption of ubiquitination and lysosomal systems may be a commonality shared by AD-vulnerable and PD-vulnerable brain tissue that begins to explain the interrelationship between the two age-related diseases.

Huntington's disease (HD)

Lysosomal dysfunction also has been found to be associated with the age-related disorder HD, also known as a polyglutamine-repeat disease. HD is an autosomal dominant disorder and, similar to previously mentioned diseases, is characterized by involuntary movements and cognitive impairment progressing to dementia. Proteins containing polyglutamine expansions can form intracellular aggregates and cause several neurodegenerative diseases. The HD mutation is a polyglutamine expansion in the N-terminal region of the protein huntingtin. Mutant huntingtin forms intraneuronal aggregates in HD brains, and expression of the mutant protein *in vitro* causes cell death (DiFiglia et al., 1997). Transport blockage as shown associated with the lysosomal dysfunction cascade is also found in HD (Li et al., 2001). Early studies in HD patients resonate the theme of a link between abnormal deposition and lysosomal dysfunction (see Cross, 1986). Along with evidence that mutant huntingtin accumulates in perinuclear regions that resemble endosomal-lysosomal organelles, a recent report confirmed that the aggregates collect in lysosomal structures (Kegel et al., 2000). The endosomal-lysosomal system appears to be one of the pathways by which endogenous huntingtin is degraded. To determine the relative involvement of other protease systems being investigated, future research should address whether experimentally-induced lysosomal dysfunction results in endogenous huntingtin accumulation.

Vicious cycles and selective vulnerability

We propose that in some age-related diseases, pathogenic processing of proteins occurs in a vicious cycle. As in HD, evidence suggests that mutant huntingtin, by slowly accumulating in lysosomes, amplifies degradation abnormalities that promote further huntingtin aggregation. Such a cycle may represent a general process in age-related diseases where expression of a pathogenic protein species alters lysosomal mechanisms, thereby causing further production of the aberrant species. This type of vicious sequence would likely facilitate deposition events, possibly involving multiple proteins that are influenced by lysosomal dysfunction. For example, α-synuclein not only accumulates in PD but also co-deposits with huntingtin in HD brains and with Aβ in AD brains (Masliah et al., 1996; Charles et al., 2000). Perhaps such deleterious cycles are initiated by fragments of APP, α-synuclein, and other proteins that can form lysosomotropic structures containing high β-sheet content (e.g., see Katz et al., 1994; Burdick et al., 1997; El-Agnaf and Irvine, 2000).

A striking example of a vicious cycle is one initiated by the $A\beta_{1-42}$ peptide and its selective uptake into vulnerable neurons (Bahr et al., 1998). Local release of secreted Aβ or the Aβ content of ruptured neurons would allow internalization by neighboring neurons. Illustrated in Figure 1 (see cycle A), lysosomes sequester the internalized Aβ as insoluble aggregates and, in so doing, promote further β-amyloidogenic processing of endogenous APP. Experimental evidence supports this progressive cycle as well as concomitant synaptic decline (Bahr et al., 1998; Yang et al., 1998; Glabe, 2001). Matching the regional vulnerability in the AD hippocampus, $A\beta_{1-42}$ is taken up exclusively by neurons in field CA1. Likewise, amyloidogenic intermediates subsequently induced by the vicious cycle were found localized to CA1 pyramidal neurons (Fig. 3A; modified from Bahr et al., 1998). The Aβ-induced CTFs are comparable to those induced by lysosomal inhibitors and to those that accumulate in neurons of AD brains (Benowitz et al., 1989; Bahr et al., 1994). Applying more exogenous Aβ showed that all hippocampal subfields as well as some dendritic zones are capable of the lysosomotropic response (Fig.

3B). These data form a link between two disparate observations in AD, i.e. extracellular Aβ levels (that lead to selective uptake) and intracellular accumulation of CTFs. They also suggest select populations of cells are especially vulnerable to a positive feedback process. Interestingly, the lysosomotropic effects could influence tau processing as well. This may form an alternative feedback loop since a recent report indicates that mutant tau disrupts lysosomes in central neurons of FTDP-17 transgenic mice (Lim et al., 2001).

The vicious cycles proposed could easily be intensified by the activation of non-lysosomal proteases that are associated with apoptotic and excitotoxic pathways. Aberrant proteolytic activity occurs with age and during many disease states (see Vanderklish and Bahr, 2000; Blomgren et al., 2001; Kim et al., 2001b), generating protein fragments that add to the workload of the lysosomal system. Caspase activation has been found to increase as a function of disease progression and to correlate with tangle formation in AD brains (Rohn et al., 2001). One of the key proteases involved in excitotoxicity is calpain, and over-activation of this protease has been linked to normal aging and AD (see reviews: Nixon, 2000; Vanderklish and Bahr, 2000). Aberrant calpain responses in the aged brain occur gradually and selectively in AD-vulnerable regions (Vicente et al., 2002). Studies have shown that calpain is activated during lysosomal disturbance (Bendiske et al., 2002), and further lysosomal disruption is produced when calpain causes proteolytic membrane damage to lysosomes (Yamashima, 2000).

Conclusion

The aging risk factor of lysosomal dysfunction, once reaching a critical level, acts as an accomplice to persuade pathogenic steps toward age-related disease states. Gradual disruption of the lysosomal system contributes to protein aggregation/deposition processes and synaptopathogenesis. *In vivo* and *in vitro* models of lysosomal dysfunction recapitulate many aspects of protein deposition diseases and, accordingly, help in the understanding of how abnormal deposits might disrupt synaptic contacts and cognition before neuronal atrophy. Supporting one hypothesis, transport failure and

the resultant down-regulated expression of synaptic components have been linked to the early events of intraneuronal deposition. Such disruption of microtubule-based transport mechanisms would explain the decline in synaptic composition and neuronal activity that is associated with age-related neurodegeneration. The evidence points to lysosomal malfunctions as playing an important part in the initial synaptic pathology. Experimentally-induced lysosomal disturbances also lead to dystrophic neurites, cytoskeletal damage, and cell death (Takauchi and Miyoshi, 1989, 1995; Hajimohammadreza et al., 1994; Okada et al., 1994; Bahr, 1995; Bendiske et al., 2002). Thus, there are strong indications that changes in the lysosomal system manipulate both early and secondary stages of age-related protein deposition diseases.

References

Amtul Z., Lewis P. A., Piper S., Crook R., Baker M., Findlay K., Singleton A., Hogg M., Younkin L., Younkin S. G., Hardy J., Hutton M., Boeve B. F., Tang-Wai D., Golde T. E. (2002) A presenilin 1 mutation associated with familial frontotemporal dementia inhibits γ-secretase cleavage of APP and notch. *Neurobiol. Dis.* **9**, 269-273.

Auer I. A., Schmidt M. L., Lee V. M., Curry B., Suzuki K., Shin R. W., Pentchev P. G., Carstea E. D., and Trojanowski J. Q. (1995) Paired helical filament tau (PHF-tau) in Niemann-Pick type C disease is similar to PHF-tau in Alzheimer's disease. *Acta Neuropathol. (Berl.)* **90**, 547-551.

Bahr B. A. (1995) Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. *J. Neurosci. Res.* **42**, 294-305.

Bahr B. A., Abai B., Gall C. M., Vanderklish P. W., Hoffman K. B., and Lynch G. (1994) Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. *Exp. Neurol.* **129**, 81-94.

Bahr B. A., Hoffman K. B., Yang A. J., Hess U. S., Glabe C. G., and Lynch G. (1998) Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. *J. Comp. Neurol.* **397**, 139-147.

Bahr B. A., Bendiske J., Brown Q. B., Munirathinam S., Caba E., Rudin M., Urwyler S., Sauter A., and Rogers G. (2002) Survival signaling and selective neuroprotection through glutamate transmission. *Exp. Neurol.* **174**, 37-47.

Bendiske J., Caba E., Brown Q. B., and Bahr B. A. (2002) Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J. Neuropathol. Exp. Neurol.* **61**, 640-650.

Benowitz L. I., Rodriguez W., Paskevich P., Mufson E. J., Schenk D., and Neve R. L. (1989) The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer disease subjects. *Exp. Neurol.* **106**, 237-250.

Bi X., Yong A.P., Zhou J., Ribak C. E., and Lynch G. (2001) Rapid induction of intraneuronal neurofibrillary tangles in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 8832-8837.

Blomgren K., Zhu C., Wang X., Karlsson J. O., Leverin A. L., Bahr B. A., Mallard C., and Hagberg H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia- ischemia: A mechanism of "pathological apoptosis"? *J. Biol. Chem.* **276**, 10191-10198.

Brunk U. and Brun A. (1972) The effect of aging on lysosomal permeability in nerve cells of the central nervous system. An enzyme histochemical study in rat. *Histochemie* **30**, 315-324.

Bucciantini M., Giannoni E., Chiti F., Baroni F., Formigli L., Zurdo J., Taddei N., Ramponi G., Dobson C. M., and Stefani M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* **416**, 507-511.

Burdick D., Kosmoski J., Knauer M. F., and Glabe C. G. (1997) Preferential adsorption, internalization and resistance to degradation of the major isoform of the Alzheimer's amyloid peptide, Aβ 1-42, in differentiated PC12 cells. *Brain Res.* **746**, 275-84.

Callahan L. M., Vaules W. A., and Coleman P. D. (1999) Quantitative decrease in synaptophysin message expression and increase in cathepsin D message expression in Alzheimer disease neurons containing neurofibrillary tangles. *J. Neuropathol. Exp. Neurol.* **58**, 275-287.

Cataldo A. M., Paskevich P. A., Kominami E., and Nixon R. A. (1991) Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10998-11002.

Cataldo A. M., Hamilton D. J., and Nixon R. A. (1994) Lysosomal abnormalities in degenerating neurons link neuronal compromise to senile plaque development in Alzheimer disease. *Brain Res.* **640**, 68-80.

Cataldo A. M., Hamilton D. J., Barnett J. L., Paskevich P. A., and Nixon R. A. (1996) Properties of the endosomal-lysosomal system in the human central nervous system: Disturbance mark most neurons in populations at risk to degenerate in Alzheimer's disease. *J. Neurosci.* **16**, 186-199.

Charles V., Mezey E., Reddy P. H., Dehejia A., Young T. A., Polymeropoulos M. H., Brownstein M. J., and Tagle D. A. (2000) α-synuclein immunoreactivity of huntingtin polyglutamine aggregates in striatum and cortex of Huntington's disease patients and transgenic mouse models. *Neurosci. Lett.* **289**, 29-32.

Cole, G.M., T.V. Huynh, and T. Saitoh. (1989) Evidence for lysosomal processing of amyloid □-protein precursor in cultured cells. *Neurochem. Res.* **14**, 933-939.

Cross A. J., Crow T. J., Dawson J. M., Ferrier I. N., Johnson J. A., Peters T. J., and Reynolds G. P. (1986)

Subcellular pathology of human neurodegenerative disorders: Alzheimer-type dementia and Huntington's disease. *J. Neurochem.* **47**, 882-889.

D'Andrea, M. R., Nagele R. G., Wang H. Y., Peterson P. A., Lee D.H. (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. *Histopathology* **38**, 120-134.

DiFiglia M., Sapp E., Chase K. O., Davies S. W., Bates G. P., Vonsattel J. P., and Aronin N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**, 1990-1993.

Ditaranto K., Tekirian T. L., and Yang A. J. (2001) Lysosomal membrane damage in soluble Aβ-mediated cell death in Alzheimer's disease. *Neurobiol. Dis.* **8**, 19-31.

El-Agnaf O. M. and Irvine G. B. (2000) Review: Formation and properties of amyloid-like fibrils derived from α-synuclein and related proteins. *J. Struct. Biol.* **130**, 300-309.

Evin G., Cappai R., Li Q. X., Culvenor J. G., Small D. H., Beyreuther K., and Masters C. L. (1995) Candidate γ-secretases in the generation of the carboxyl terminus of the Alzheimer's disease β A4 amyloid: Possible involvement of cathepsin D. *Biochemistry* **34**, 14185-14192.

Fassbender K., Simons M., Bergmann C., Stroick M., Lutjohann D., Keller P., Runz H., Kuhl S., Bertsch T., von Bergmann K., Hennerici M., Beyreuther K., and Hartmann T. (2001) Simvastatin strongly reduces levels of Alzheimer's disease β-amyloid peptides Aβ 42 and Aβ 40 *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 5856-5861.

Glabe C. (2001) Intracellular mechanisms of amyloid accumulation and pathogenesis in Alzheimer's disease. *J. Mol. Neurosci.* **17**, 137-145.

Golde T. E., Estus, S., Younkin L. H., Selkoe D. J., and Younkin S. G. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* **255**, 728-730.

Gotz J., Chen F., van Dorpe J., and Nitsch R. M. (2001) Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Aβ 42 fibrils. *Science* **293**, 1491-1495.

Haass C., Koo E. H., Mellon A., Hung A. Y., and Selkoe D. J. (1992) Targeting of cell-surface β-amyloid precursor protein to lysosomes: Alternative processing into amyloid-bearing fragments. *Nature* **357**, 500-503.

Haass C., Hung A. V., Schlossmacher M. G., Teplow D. B., Selkoe D. J. (1993) β-amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J Biol Chem.* **268**, 3021-3024.

Hajimohammadreza I., Anderson V. E. R., Cavanagh J. B., Seville M. P., Nolan C. C., Anderton B. H., and Leigh P. N. (1994) β-Amyloid precursor protein fragments and lysosomal dense bodies are found in rat brain neurons after ventricular infusion of leupeptin. *Brain Res.* **640**, 25-32.

Hardy J., Duff K., Hardy K. G., Perez-Tur J., and Hutton M. (1998) Genetic dissection of Alzheimer's disease and related dementias: Amyloid and its relationship to tau. *Nat. Neurosci.* **1**, 355-358.

Hempen B. and Brion J. P. (1996) Reduction of acetylated α-tubulin immunoreactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* **55**, 964-972.

Hsia A. Y., Masliah E., McConlogue L., Yu G. Q., Tatsuno G., Hu K., Kholodenko D., Malenka R. C., Nicoll R. A., and Mucke L. (1999) Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3228-3233.

Ikeda K., Akiyama H., Arai T. et al. (1998) Alz-50/Gallyas-positive lysosome-like intraneuronal granules in Alzheimer's disease and control brains. *Neurosci. Lett.* **258**, 113-116.

Ikeda K., Akiyama H., Arai T., Kondo H., Haga C., Tsuchiya K., Yamada S., and Murayama S. (2000) Neurons containing Alz-50-immunoreactive granules around the cerebral infarction: Evidence for the lysosomal degradation of altered tau in human brain? *Neurosci. Lett.* **284**, 187-189.

Ivy G. O. (1992) Protease inhibition causes some manifestations of aging and Alzheimer's disease in rodent and primate brain. *Ann. N. Y. Acad. Sci.* **674**, 89-102.

Iwata N., Tsubuki S., Takaki Y., Shirotani K., Lu B., Gerard N. P., Gerard C., Hama E., Lee H. J., and Saido T. C. (2001) Metabolic regulation of brain Aβ by neprilysin. *Science* **292**, 1550-1552.

Kamal A., Almenar-Queralt A., LeBlanc J. F., Roberts E. A., and Goldstein L. S. (2001) Kinesin-mediated axonal transport of a membrane compartment containing β-secretase and presenilin-1 requires APP. *Nature* **414**, 643-648.

Katz M. L., Christianson J. S., Norbury N. E., Gao C. L., Siakotos A. N., and Koppang N. (1994) Lysine methylation of mitochondrial ATP synthase subunit c stored in tissues of dogs with hereditary ceroid lipofuscinosis. *J. Biol. Chem.* **269**, 9906-9911.

8

Kegel K. B., Kim M., Sapp E., McIntyre C., Castano J. G., Aronin N., and DiFiglia M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J. Neurosci.* **20**, 7268-7278.

Kikugawa K., Beppu M., Kato T., Yamaki S., and Kasai H. (1994) Accumulation of autofluorescent yellow lipofuscin in rat tissues estimated by sodium dodecylsulfate extraction. *Mech. Ageing Dev.* **74**, 135-148.

Kim J.-H., Anwyl R., Suh Y.-H., Djamgoz M. B. A.,and Rowan M. J. (2001a) Use-dependent effects of amyloidogenic fragments of β-amyloid precursor Protein on synaptic plasticity in rat hippocampus *in vivo*. *J. Neurosci.* **21**, 1327-1333.

Kim Y. J., Yi Y., Sapp E., Wang Y., Cuiffo B., Kegel K. B., Qin Z. H., Aronin N., and DiFiglia M. (2001b) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12784-12789.

Klafki H. W., Abramowski D., Swoboda R., Paganetti P. A., and Staufenbiel M. (1996) The carboxyl termini of β amyloid peptides 1-40 and 1-42 are generated by distinct γ-secretase activities. *J. Biol. Chem.* **271**, 28655-28659.

Koo E. H. and Squazzo S. (1994) Evidence that production and release of amyloid β-protein involves the endocytic pathway. *J Biol Chem.* **269**, 17386-17389.

Laszlo L., Lowe J., Self T., Kenward N., Landon M., McBride T., Farquhar C., McConnell I., Brown J., and Hope J. (1992) Lysosomes as key organelles in the pathogenesis of prion encephalopathies. *J. Pathol.* **166**, 333-341.

Lewis J., Dickson D. W., Lin W. L., Chisholm L., Corral A., Jones G., Yen S. H., Sahara N., Skipper L., Yager D., Eckman C., Hardy J., Hutton M., and McGowan E. (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**, 1487-1491.

Li H., Li S. H., Yu Z. X., Shelbourne P., and Li X. J. (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *J. Neurosci.* **21**, 8473-8481.

Lim F., Hernandez F., Lucas J. J., Gomez-Ramos P., Moran M. A., and Avila J. (2001) FTDP-17 mutations in tau transgenic mice provoke lysosomal abnormalities and tau filaments in forebrain. *Mol. Cell. Neurosci.* **18**, 702-714.

Loftus S. K., Morris J. A., Carstea E. D., Gu J. Z., Cummings C., Brown A., Ellison J., Ohno K., Rosenfeld M. A., Tagle D. A., Pentchev P. G., and Pavan W. J. (1997) Murine model of Niemann-Pick C disease: Mutation in a cholesterol homeostasis gene. *Science* **277**, 232-235.

Masliah E. and Licastro F. (2000) Neuronal and synaptic loss, reactive gliosis, microglial response, and induction of the complement cascade in Alzheimer's disease, in Neurodegenerative Dementias (Clark C. M. and Trojanowski J. Q., eds), pp. 131-146. McGraw-Hill, New York.

Masliah E., Iwai A., Mallory M., Ueda K., and Saitoh T. (1996) Altered presynaptic protein NACP is associated with plaque formation and neurodegeneration in Alzheimer's disease. *Am. J. Pathol.* **148**, 201-210.

Mathews P. M., Guerra C. B., Jiang Y., Grbovic O. M., Kao B. H., Schmidt S. D., Dinakar R., Mercken M., Hille-Rehfeld A., Rohrer J., Mehta P., Cataldo A. M., and Nixon R. A. (2002) Alzheimer's disease-related overexpression of the cation-dependent mannose 6-phosphate receptor increases Aβ secretion: Role for altered lysosomal hydrolase distribution in β-amyloidogenesis. *J. Biol. Chem.* **277**, 5299-5307.

McPhie, D. L., Golde T., Eckman C. B., Yager D., Brant J. B., and Neve R. L (2001) β-Secretase cleavage of the amyloid precursor protein mediates neuronal apoptosis caused by familial Alzheimer's disease mutations. *Mol. Brain Res.* **97**, 103-113.

Mesulam M. M. (1999) Neuroplasticity failure in Alzheimer's disease: Bridging the gap between plaques and tangles. *Neuron* **24**, 521-529

Mielke J. G., Murphy M. P., Maritz J., Bengualid K. M., and Ivy G. O. (1997) Chloroquine administration in mice increases β-amyloid immunoreactivity and attenuates kainate-induced blood-brain barrier dysfunction. *Neurosci. Lett.* **227**, 169-172.

Mills J. and Reiner P. B. (1999) Regulation of amyloid precursor protein cleavage. *J. Neurochem.* **72**, 443-460.

Mucke L., Masliah E., Yu G. O., Mallory M., Rockenstein E. M., Tatsuno G., Hu K., Kholodenko D., Johnson-Wood K., and McConlogue L. (2000) High-level neuronal Expression of Aβ1-42 in wild-type human amyloid protein precursor transgenic mice: Synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050-4058.

Nakamura Y., Takeda M., Suzuki K., Morita K., Tada K., Hariguchi S., and Nishimura R. (1998) Lysosome instability in aged rat brain. *Neurosci. Lett.* **97**, 215-220.

9

Nixon R. A. (2000) A "protease activation cascade" in the pathogenesis of Alzheimer's disease. *Ann. N. Y. Acad. Sci.* **924,** 117-131.

Nixon R. A., Mathews P. M., and Cataldo A. M. (2001) The neuronal endosomal-lysosomal system in Alzheimer's disease. *J. Alzheimer's Diease* **3,** 97–107.

Okada M., Miyake T., Kitamura T., Kawasaki K., and Mizushima Y. (1994) Anti-ubiquitin immunoreactivity associates with pyramidal cell death induced by intraventricular infusion of leupeptin in rat hippocampus. *Neurosci. Res.* **19,** 59-66.

Oster-Granite M. L., McPhie D. L., Greenan J., and Neve R. L. (1996) Age-dependent neuronal and synaptic degeneration in mice transgenic for the C terminus of the amyloid precursor protein. *J. Neurosci.* **16,** 6732-6741.

Oyama F., Murakami N., and Ihara Y. (1998) Chloroquine myopathy suggests that tau is degraded in lysosomes: Implication for the formation of paired helical filaments in Alzheimer's disease. *Neurosci. Res.* **31,** 1-8.

Rohn T. T., Head E., Su J. H., Anderson A. J., Bahr B. A., Cotman C. W., and Cribbs D. H. (2001) Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *Am. J. Pathol.* **158,** 189-198.

Selkoe D. J. (2001a). Alzheimer's disease: Genes, proteins and therapies. *Physiol. Rev.* **81,** 742-761.

Selkoe D. J. (2001b) Clearing the brain's amyloid cobwebs. *Neuron* **32,** 177-180.

Skovronsky D. M., Pijak D. S., Doms R. W., and Lee V. M. (2000) A distinct ER/IC γ-secretase competes with the proteasome for cleavage of APP. *Biochemistry* **39,** 810-817.

Small D. H., Mok S. S., and Bornstein J. C. (2001) Alzheimer's disease and Aβ toxicity: From top to bottom. *Nat. Rev. Neurosci.* **2,** 595-598.

Spillantini M. G., Schmidt M. L., Lee V. M., Trojanowski J. Q., Jakes R., and Goedert M. (1997) α-synuclein in Lewy bodies. *Nature* **388,** 839-840.

Stefanis L., Larsen K. E., Rideout H. J., Sulzer D., Greene L. A. (2001) Expression of A53T mutant but not wild-type α-synuclein in PC12 cells induces alteration of the ubiquitin-dependent degradation system, loss of dopamine release and autophagic cell death. *J Neurosci.* **21,** 9549-9560.

Takauchi S. and Miyoshi K. (1989) Degeneration of neuronal processes in rats induced by a protease inhibitor, leupeptin. *Acta Neuropathol. (Berl.)* **78,** 380-387.

Takauchi S. and Miyoshi K. (1995) Cytoskeletal changes in rat cortical neurons induced by long-term intraventricular infusion of leupeptin. *Acta Neuropathol. (Berl.)* **89,** 8-16.

Terry R. D., Masliah E., Salmon D. P., Butters N., Deteresa R., Hill R., Hansen L. A., Katzman R. (1991) Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* **30,** 572-580.

Vanderklish P. W. and Bahr B. A. (2000) The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Int. J. Exp. Pathol.* **81,** 323-339.

Vicente J. S., Munirathinam S., and Bahr B. A. (2002) Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age. *J. Mol. Biol. Biotech.* **3,** 1-10.

Walkley S. U. (1998) Cellular pathology of lysosomal storage disorders. *Brain Pathol.* **8,** 175-193.

Walsh D. M., Klyubin I., Fadeeva J. V., Cullen W. K., Anwyl R., Wolfe M. S., Rowan M. J., and Selkoe D. J. (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo. Nature* **416,** 535-539.

Yamashima T. (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog. Neurobiol.* **62,** 273-295.

Yang, A. J., Knauer M., Burdick D. A., and Glabe C. (1995) Intracellular Aβ 1-42 aggregates stimulate the accumulation of stable, insoluble amyloidogenic fragments of the amyloid precursor protein in transfected cells. *J. Biol. Chem.* **270,** 14786-14792.

Yang A. J., Chandswangbhuvana D., Margol L., and Glabe C. G. (1998) Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Aβ1-42 pathogenesis. *J. Neurosci. Res.* **52,** 691-698.

TABLE 1. Evidence of lysosomal abnormalities is associated with the mental deterioration characteristic of early-onset and age-related diseases.

Early-Onset Pathologies	Age-Related Pathologies
Tay-Sach's disease	Alzheimer's disease
juvenile neuronal ceroid lipofuscinosis	FTDP-17
Niemann-Pick disease	Parkinson's disease
Sandhoff's disease	Lewy body dementia
Sanfillippo B syndrome	Huntington's disease

FIGURE LEGENDS

Figure 1. Possible contributions of age-related lysosomal disturbances to AD-type pathogenesis.

Figure 2. Lysosomal disturbance leads to synaptic pathology. Anti-synaptophysin staining in hippocampal slice cultures was compared before (A) and after 6 days of lysosomal dysfunction (B), which was experimentally-induced with chloroquine. The presynaptic marker was assessed by threshold digitization at high magnification (in red). The density of organized terminals on stratum radiatum dendrites was reduced from 10-15 μm^{-1} in control tissue to 1-2 μm^{-1} following lysosomal dysfunction. View-field width: 60 μm.

Figure 3. Internalization of $A\beta_{1-42}$ initiates amyloidogenic processing in vulnerable neurons. (A) Cultured hippocampal slices were treated daily with the $A\beta_{1-42}$ peptide for a week. Sections were then immunostained with affinity-purified anti-Aβ antibodies that do not label monomeric Aβ but recognize intracellular amyloidogenic fragments (developed by C. Glabe, University of California, Irvine). A distinct zone of pyramidal neurons (arrow) was labeled in field CA1. (B) Increasing the Aβ dose five-fold caused widespread induction of the amyloidogenic response in CA1, CA3, and the stratum granulosum (sg). Immunopositive dendrites and terminals also were evident in the stratum radiatum (sr), stratum lacunosum-moleculare (slm), and the molecular layer of the dentate gyrus (ml). View-field width: 3.5 mm.

Neuronal Atrophy

Age-Related Processes

Aberrant Proteolytic Response

Lysosomal Alterations

Synaptic Deterioration

A

Reduced Neurotransmission

Disrupted Plasticity Mechanisms

Transport Disturbances

NFT

Tau ⇌ pTau

Hyperphos. Tau

?

A
CA1
DG
CA3

B
CA1
sr
slm
−ml
sg
CA3